Biochemistry

AND

Human Metabolism

BY

BURNHAM S. WALKER, M.D., Ph.D.
Professor of Biochemistry
Boston University School of Medicine

WILLIAM C. BOYD, Ph.D.
Professor of Immunochemistry
Boston University School of Medicine

ISAAC ASIMOV, Ph.D.
Assistant Professor of Biochemistry
Boston University School of Medicine

WITH A FOREWORD BY

JOHN T. EDSALL, M.D.
Professor of Biological Chemistry
Harvard University



BALTIMORE
THE WILLIAMS & WILKINS COMPANY
1952

Composed and Printed for
THE WILLIAMS & WILKINS COMPANY
BY
THE WAVERLY PRESS INC
BALTIMORE 2 MD U.S.A

Foreword

This book will certainly win adequate recognition without the need of any foreword from a colleague However, I am glad that Drs Walker Boyd and Asimov have given me the opportunity to read it in manuscript and to record the belief that it has a freshness and vitality in its general outlook and in the pattern of the presentation which give it a distinctive place among all the texts of biochemistry for medical students of which I am aware Naturally as a protein chemist I must express my enthusiasm for the decision of the authors to put proteins first in the prime position where they ought to be Moreover they have succeeded in saying a good many true and important things about proteins and amino acids which have not yet found their way into most of the elementary books

It is unnecessary here to comment in detail on the rest of the book the general organization of which seems to me admirable One may call particular attention to the discussion of genetics for the interpenetration of biochemistry and genetics has been one of the great scientific developments of the last fifteen years Moreover medical students generally know far too little about genetics, it is a vital subject for the practicing physician to know today and it will become even more vital in the generation to come

An important feature of the book to me is its combined treatment of fundamental biochemistry and many of the clinical applications This should help to awaken more medical students to the potential clinical significance of what they are studying even in the more abstract portions of biochemistry and to remind the student in the clinic later on of the foundations of the methods which he is applying The theory and practical applications are interwoven so that each reinforces the other For this and for many other contributions one may confidently wish and expect that this book will serve its purpose well

John T Edsall

Preface

The events and motivations that led to the construction of this book were neither few nor simple Of them however one and only one need be of any concern to the reader That refers to our intention to prepare a book on biochemistry intended primarily for the medical student This intention has constantly been in our minds and has dictated our every decision in the matter of what to include in the book and how to include it

A biochemistry text for medical students must of necessity differ from a biochemistry text for biochemists in certain important particulars Much organic chemistry may be sacrificed with profit considering that the space thus saved may be utilized for greater detail in clinical applications of biochemistry The classical order of topics can be and has been altered in order that proteins may be taken up first as that group of chemical substances is of prime importance to medical biochemistry The chemistry of carbohydrates and lipids can be and has been taken up as an introduction to tissue chemistry Human biochemistry has been emphasized almost to the exclusion of other branches of biochemistry

We have not hesitated to stress the current uncertainties in biochemical theory In fact we have gone out of our way to do so and where one or more of the authors have felt sufficiently venturesome have even taken sides This is a deliberate attempt to impress upon the student that biochemistry is not a closed science that can make statements with finality which need only be memorized to remain in good repute for a lifetime Rather we show biochemistry to be a growing expanding science which particularly today sometimes changes with bewildering rapidity The physician therefore must understand that the biochemistry he is taught is merely the basis for the biochemistry he must continually teach himself as the years pass

No attention has been given to historical priority in choosing articles for citation The choice has been in most instances in favor of reviews or of relatively recent papers which themselves cite the original work

That the book may contain errors we are painfully aware To those who point such out to us we will be grateful

We wish to express our thanks to the numerous friends and colleagues both at Boston University and elsewhere who have read and criticized portions of this manuscript

One of us (W C B) wishes to express his gratitude for permission from Little, Brown and Company to make use of certain material which appeared in his book, *Genetics and the Races of Man*

Another (I A) wishes to thank the National Cancer Institute, National Institutes of Health, U S Public Health Service for its support from teaching and research grants over the period during which this book was written

The preparation of the typescript was done by Miss Georgiana M Curtin

B S W
W C B
I A

Contents

Part VI Appendices

PART I

Structure

CHAPTER 1

Proteins and Amino Acids

Just as it is necessary when beginning the study of medicine to learn first of all the gross anatomy of the human body, so in beginning the study of the chemistry of life processes it is necessary to study first of all the structural chemistry of living matter. The characteristic material of living matter is called *protein*. The term was first applied to complex nitrogenous substances found in plant and animal tissues by Mulder in 1838. The word comes from Greek roots meaning preeminence. If we compare a living cell with a machine, then we can compare the protein with the steel and brass out of which the engine is constructed. Other substances to be discussed later, are to be found in living tissue and many of them have indispensable functions there, but the material which is most essential to life itself—indeed which is life itself—is the tissue protein.

All living cells contain protein, carbohydrate, lipids, water, and inorganic ions, linked together in various ways to form compounds and complexes. Most of our chemical study of these materials comes only after the cells have been taken apart and the individual constituents isolated. The method of taking the cell apart often influences the result. For instance, if we represent the various constituents of a cell by the letters, A, B, C, D, and assume that by rough treatment of some sort we split the cell up into fragments which can be dealt with chemically, it is easy to see that we might come out with complexes such as AB, BC, ABC, CD, CDE, depending on the methods used and the severity of the treatment. This does in fact happen and it is always difficult to make sure that what has been isolated is a reproducible material which actually represents a constituent of the cell, for if at one time we obtain AB from the cell and another time BC or ABC, our chemical analyses in the two instances will be somewhat contradictory, or at least inconsistent. Indeed, since there is every reason to suspect that the protein in the cell is rarely if ever free the idea of pure protein is in itself an idealization and represents something which, probably, is not to be found in the average tissue. But, like many other abstract ideas it is a very useful one, and we shall

find it very valuable to examine the properties of purified proteins and to try to apply this knowledge to the behavior of the living cell

That the concept of protein is not entirely an imaginary one has been demonstrated by the isolation of proteins with reproducible properties from various types of tissue It is true that the situation is not as simple as it was thought to be by Mulder, who believed that there was only one protein which combined with sulfur or phosphorus or both to give various compounds, and that it was these which were present in the tissues of plants and animals Indeed, we know that there are a very large number of proteins although we have not obtained very many of them in a pure state It is not usually to be expected that even a single organ will contain but one sort of protein, for diversity of chemical structure is required by the manifold functions performed by most of the organs of the human body We can not state, for instance that there is such a thing as a "kidney protein" because the kidney contains proteins of various types So do most of the tissues However, there are proteins more or less characteristic of certain types of tissues and our ability to isolate them in more or less pure form justifies our naming them for the tissue from which they come or for some special property or activity which they exhibit

DEFINITION OF PROTEIN

It is at once difficult and easy accurately to define a protein It is easy in the sense that it is not difficult to give the reader a fairly clear idea of what we mean by the word It is difficult in the sense that we find difficulty in being absolutely precise about our definition Suppose we simply say for the time being that proteins are large molecules of molecular weight of the order of several thousand to several million occurring in the tissues of plants and animals and containing carbon, hydrogen oxygen, nitrogen, and sometimes other elements, and constructed largely from *amino acids*, which will be discussed later in this chapter This definition serves to differentiate proteins from all the other compounds we shall study in this book

PROPERTIES OF PROTEINS

In discussing the properties of the proteins we shall want to say something about their behavior *in vivo*—that is, in the intact cell—and *in vitro*—that is, as they behave in the laboratory after we have isolated them

Lability

One of the most striking characteristics of the proteins is that they are extremely sensitive to change and it is difficult to keep them from change during chemical manipulation Probably one of the first and most serious

changes which takes place in a cell when the organism of which it forms a part has died is alteration in the constituent proteins These changes soon reach the stage where they are irreversible and the cell is then itself dead As an example of this lability we may consider the properties of the egg albumin, which forms the principal protein of the white of the hen's egg This protein can be isolated from the egg and obtained in crystalline form, but as a rule only if the egg is less than 24 hours old If the egg is older than this great difficulty is experienced in trying to crystallize the protein The egg is still viable, it will still hatch but nevertheless something has happened to the albumin during the first twenty four hours the egg has been outside the body of the hen And keeping the egg at body temperature, instead of arresting this process, merely accelerates it After the albumin has been crystallized, it will keep in the form of crystals covered with mother liquor for a considerable time, but nevertheless at room temperature or even at icebox temperatures slow changes take place, so that the solubility of the crystals in water gradually becomes less, and after a few years they may become completely insoluble, showing that even when the protein is in the form of crystals and relatively pure, something has happened to it Crystals of the blood protein hemoglobin, even when kept in the icebox, lose their characteristic properties even more rapidly than does egg albumin

In order to isolate proteins with as little damage as possible the temperature must be kept low—lower than body temperature whenever conditions permit, otherwise random thermal agitation of the atoms will cause some denaturation Also, enzymes which are originally present in the impure preparation may begin to act on the protein before they are separated from it The pH[1] should be maintained as near that of the environment of the native protein as possible The dielectric constant of the medium should be kept as high as possible which means that other things being equal an aqueous medium is best Organic solvents such as alcohol should be used only with great caution and preferably at low temperatures They appear to damage certain proteins only slightly but may rapidly damage others The use of high salt concentrations to precipitate proteins, though traditional, should be avoided when possible In some cases this seems to result in very slight damage but ultimately damage always does result Some proteins are damaged immediately beyond repair by this procedure

Solubility

Some proteins are insoluble in all ordinary solvents, others are more or less soluble in various mixtures of water and other compounds Pure water will dissolve some proteins, in other cases it acts as a better solvent if

[1] The symbol pH is defined on page 19

salts are present Some proteins will dissolve best in mixtures of wat and less polar solvents such as alcohol In general, the more polar th solvent the greater its power of dissolving proteins, which suggests th the protein is itself polar in nature If sufficient salt especially a salt suc as ammonium sulfate is dissolved in a protein solution, the protein be comes less soluble and most proteins are completely precipitated Ther are certain general rules about the solubility of proteins which may no be stated

1 A protein is least soluble in the neighborhood of its isoelectric point The term *isoelectric point* will be defined on page 17 The pH of mini mum solubility varies with the nature and concentration of the salt which is used Unless the salt concentration is very dilute the pH of minimum solubility is generally found to be somewhat different from the true iso electric point of the protein

2 Solubility of proteins in water without salt varies a great deal Serum albumin dissolves in water readily and it seems to be miscible with water in all proportions Other proteins are soluble only if salt is present, some like edestin from hempseed, require concentrations of neutral salt of the order of 5 per cent to get them into solution

3 The solubility of a protein in water or other solvents depends upon the nature of the amino acids of which it is composed Thus proteins rich in non polar groups such as paraffin side chains, benzene rings, or pyrroli dine rings tend to dissolve better in alcohol water mixtures than in water whereas those poor in non polar groups but rich in polar (electrically charged) groups tend to be precipitated even by small amounts of alcohol or acetone

4 Proteins which are insoluble in water but have large numbers of charged groups become more soluble in the presence of neutral salts or other dipolar ions

5 Proteins are usually more soluble when combined with acids or bases than in the neutral state This will be discussed immediately below

6 Formation of salts between proteins and another protein or between a protein and ion may result in compounds which are more or less soluble Thus protamines form a compound with insulin which is less soluble than either protamine or insulin Protamines also form insoluble salts with casein

Amphoteric Behavior of Proteins

Most proteins can behave either as acids or bases and are thus called amphoteric Consequently it is possible to dissolve these proteins either in dilute acid or dilute alkali forming a salt in either case This amphoteric behavior of proteins is of great importance to understanding other proper

ties in general For instance whether a protein combines with an anion or a cation depends upon the pH of a solution or in other words, on which side of the isoelectric point of the protein we find ourselves This was well shown in the classical experiment of Jacques Loeb, described on page 18

The amphoteric behavior of proteins is due to the presence of acidic and basic groups in their molecules (p 28) Some of these groups are ordinarily charged, positively or negatively as the case may be, and thus account for the presence on the surface of the molecule of fixed charges Except at the isoelectric points, the positive and negative charges do not usually balance exactly, so the molecule has an over all net charge which is positive or negative, depending on pH The distributions of the two sorts of charges are seldom the same, so negative charges will predominate in one part of the molecule and positive charges in another The effect of this is the same as the localization of a positive charge of varying magnitude on one part of the molecule and a negative charge on another part This causes the protein molecule, even at the isoelectric point, to behave like an electric dipole Therefore, protein molecules in solution will orient themselves in an electric field

A dipolar ion will tend to orient itself in an electric field with the end which is predominantly negative pointing towards the positive pole and the positive end towards the negative pole

The dielectric constant of a solution can be interpreted as being almost entirely a measure of the number of molecules oriented by the electric field This orientation is hindered by frictional forces which vary with the size and shape of the molecule Hence, a study of the dielectric constant of a protein solution subjected to alternating electric fields of various frequencies gives information about the physical characteristics of the protein molecules (21)

Ability to Form Complexes

Proteins, having a fair number of charged groups which may be of either sign, can and do form complexes of various sorts Some of them form insoluble salts with anions or with cations For instance, the vegetable protein, edestin, is not only relatively insoluble itself but forms a relatively insoluble hydrochloride Some proteins which form soluble sodium or potassium salts form insoluble calcium salts Others form insoluble zinc salts A large number of substances form insoluble compounds with nearly all proteins and are thus used as tests for proteins Among these we may list tannic acid, picric acid, phosphotungstic acid, and trichloracetic acid These will be mentioned under tests for proteins The process of tanning leather seems to be a process of formation of insoluble complexes with various agents such as tannic acid, chromic acid, and so forth Proteins may also form

insoluble complexes with other proteins A very well known *example is the* complex of insulin and the simple protein, protamine The combination of insulin and protamine is used clinically because the insolubility of the complex results in its liberating insulin into the circulation more slowly than if the hormone were injected by itself

EXTRACTION AND PURIFICATION OF PROTEINS

In order to make chemical studies of proteins, it is necessary to extract them from the tissues in which they occur and follow this by purification procedures In the case of blood, the proteins of the plasma are already in solution and it is only necessary to add something to prevent the clotting of the blood, if we wish to study also the fibrinogen, or to take the serum which separates from the clot in case we are not interested in the fibrinogen But blood is a rather special tissue, although one of great importance, and it is somewhat easier to study than most of the others If we have tissue consisting of cells, connective tissue, fat, and so forth, and wish to study one of the proteins contained therein, one of our first problems is how to break up the cells so as to get out the proteins To break up the cells of the tissue, several methods are available Alternate freezing and thawing will usually accomplish it, or grinding with sand in some sort of mill, or the action of intense sound waves of high frequency ("ultrasonic vibration") In a given case one of these may have some advantage over the other It is probable that none is quite ideal In some cases simple *mincing of the tissue, followed by extraction with dilute salt solution* will extract a large amount of protein present This is perhaps the mildest of the methods of extraction

The purification procedure: concept of purification It is somewhat more difficult to establish the purity of a protein than in the case of simpler chemical compounds, although even in these cases it is not always as easy as might be supposed In a case of simple organic compounds which can *be crystallized readily*, it is usual to repeat crystallization until one or several of the properties of the substance remain unchanged after further crystallization For instance, it is customary to follow the melting point and crystallize until the product no longer changes its melting point after further crystallizations Elementary analyses (C, H, N, and so forth) also help one to follow the degree of purity attained *But some compounds such*

products of alcoholic fermentation Distillation to consta[illegible] is usually a good method of fractionating liquids and obtaining pure components But the reader will recall that if this is attempted in the case of

mixtures of ethyl alcohol and water, the constant boiling mixture as finally obtained contains between 95 and 96 per cent alcohol and no further distillation will improve the purity because a *mixture* with a constant boiling point has been obtained Other methods must be relied upon to remove the rest of the water A similar situation can arise in crystallization if compounds are formed which separate out in the form of crystals and the solubility of the compound is less than that of either component There is good reason to think that this does often happen with protein materials The usual definition of 'pure" for an organic substance is a substance which consists of a single molecular species—that is, all of its molecules are exactly alike This concept, though satisfactory enough for most organic compounds, especially those dissolved in non polar solvents, is not at all satisfactory when applied to proteins—even ideally In fact there is very good evidence in many cases that it is not so Repeatedly crystallized material can be shown to be non homogeneous In the case of proteins we are inclined to rely upon physical and relatively crude criteria such as solubility, the sedimentation constant as determined in the ultracentrifuge (see below), and the electrophoretic mobility as determined in the Tiselius electrophoresis apparatus It is desirable that supposedly pure protein should conform to the phase rule of solubility, that is, if the amount which goes into solution is plotted against the amount added, a straight line of definite slope should be obtained up to a certain point after which this line abruptly becomes horizontal and does not change its slope thereafter The point at which the line changes its slope, of course, represents the point at which saturation is obtained (See figure 1)

It is natural to suppose that all the molecules of a protein which fulfills a single function in a single tissue are exactly alike But this is not necessarily so Human serum albumin, after careful separation and repeated crystallization, is homogeneous electrophoretically, ultracentrifugally, and immunologically But amino acid analysis suggests that there is only half as much tryptophane present as would be needed for one tryptophane residue to be present in each molecule

Even when there is no difference in structure between the different molecules of a purified protein, it is unlikely that all of them are at any one time in the same state Thus Cohn and Edsall (3) calculate that in the case of the hemoglobin studied by them at pH 6 4, the isoelectric point, only 22 4 per cent of the molecules possessed zero net charge They estimated that 21 2 per cent possessed a net charge of -1, and 0 03 per cent, a net charge of -7

Salting out. After the protein has been obtained in the form of a solution from the cells, the next procedure is to purify it A number of different possibilities present themselves, some better than others One of the older

methods consists in what is called 'salting out"—that is, the addition of a sufficient concentration of a neutral salt such as *sodium chloride, magnesium chloride*, or ammonium sulfate, the latter being the one most commonly used to precipitate the protein and throw it out of solution We may consider that salting out depends on the monopolization of so much of the water by the more polar salt that not enough is free to keep the protein in *solution If the precipitate is then filtered off*, it may be redissolved and the process repeated This will often result in considerable purification because small amounts of other proteins, particularly if they are relatively soluble, will be left behind in the filtrate so that eventually a relatively pure protein

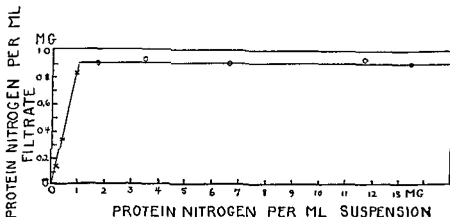


FIG 1 Solubility of crystalline chymotrypsinogen in one quarter saturated ammonium sulfate at 10 C in the presence of increasing quantities of chymotrypsinogen in the solid phase (Northrop)

preparation will result Crystallization can be achieved in many cases by bringing salt concentrations to the right concentration and adjusting the pH to a value in the vicinity of the isoelectric point Such methods have allowed the crystallization of egg albumin, serum albumin, and various other proteins However, the addition of high concentrations of salts to proteins is *not without danger and in many cases*, without doubt, results in denaturation, which may be mild or in some cases pronounced

It has been shown that the salting out of a protein depends upon the character of the protein and also upon the particular neutral salt which is used The characteristics of salting out with ammonium sulfate for a number of proteins are shown in figure 2 taken from Cohn (3) and for a number *of salts in figure 2a*

Crystallization. It has been possible to purify many proteins by crystallization The most successful of the early procedures were based on the prin

ciple of lowering the solubility of the protein by adding an amount of a polar salt such as ammonium sulfate which brings the protein to the verge of precipitation (impurities of lower solubilities are removed by a prelim inary step) Then the pH is brought down by the addition of buffer or dilute acetic acid to the vicinity of the isoelectric point or until a slight cloudiness appears This cloudiness under favorable conditions develops into a deposit of protein crystals in the course of 24 hours or so Sometimes it is better to adjust the pH and add saturated ammonium sulfate solution

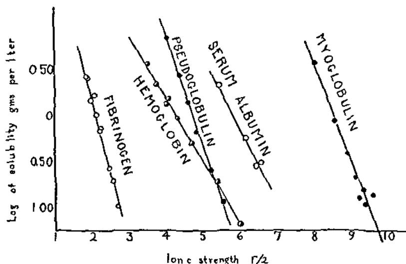


Fig 2 Solubility of five different proteins in ammonium sulfate solutions of various ionic strengths $\Gamma/2$ = ionic strength = $1/2 \Sigma c z_i^2$ where c is the actual concentration (molality) of each ion and z is its valence Note linear relation between log of solubility and ionic strength

until cloudiness begins to develop By modifications of these methods many enzymes have been crystallized (16)

The use of these high salt concentrations is not without danger to the protein Many proteins may be crystallized at lower temperatures by using alcohol water mixtures as the solvent (Cohn and Hughes) Hughes later discovered that the presence of chloroform or, especially aliphatic alcohols such as decanol made crystallization easier In some cases the use of decanol or some such substance was absolutely essential and made possible the crystallization of proteins which have never been crystallized from solutions of salts such as ammonium sulfate The damage to the protein in this method seems to be very slight

Proteins from different sources vary in the ease with which they crystallize The albumin *from the white of the hen s fresh* egg is relatively easy to crystallize, but that from duck s egg is very difficult Crystallized proteins show evidence of being purer than amorphous preparations, but are not always *completely homogeneous even after* repeated crystallization (11, 17a)

Dialysis. Another means of purifying proteins depends upon the fact that

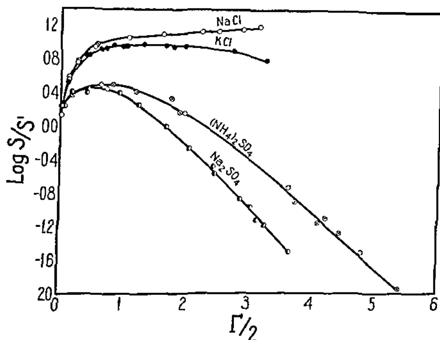


FIG 2a Solubility of hemoglobin in salt solutions of various ionic strengths S/S stands for the ratio of the solubility at the indicated ionic strength to the solubility *in distilled water Note initial increase in solubility (salting in) followed by* subsequent larger decrease (salting out)

they will not diffuse through fine membranes which possess pores large enough to admit the passage of smaller molecules, ordinary ions, or even

water and the other only in salt solutions In that case dialysis may be continued to the point where the salt concentration is so low that the second protein is precipitated whereas the water soluble protein remains in solu

tion Filtration then allows their separation This method of purification has been effective, for instance with the hemocyanins (see page 42), and Dhéré succeeded in crystallizing a number of hemocyanins by slow dialysis

In order to free proteins completely from all impurities except ions with which they are in chemical combination it is usually necessary to employ electrodialysis, in which case the movement of the ions through the membrane is speeded up and facilitated by the use of an electric potential between the inside and outside of the membrane Here too there is danger of denaturation and the process must be used with caution especial attention being directed to avoiding local heating effects

Adsorption Another method which has been employed to purify proteins is the method of adsorption The protein is adsorbed on a suitable material such as diatomaceous earth or powdered aluminum hydroxide and after this material has been washed free of non adsorbed or loosely adsorbed impurities the protein is removed (eluted) by suitable means This method was used in the early days of protein chemistry in attempts to purify enzymes (p 200) and antibodies (p 724) It gave very small yields however, and in many cases seems to have produced some denaturation In fact, any process which brings a protein to an interface be it solid liquid or liquid gas is risky

Ultracentrifugation Another method of separating proteins depends upon the use of the ultracentrifuge which is discussed immediately below Proteins which differ to any great extent in molecular weight can be separated by this method the larger molecules being deposited first on the bottom of the centrifuge tube However only relatively small amounts can be handled by this technique and it is primarily an analytical and not a manipulative tool

The ultracentrifuge as its name implies is a centrifuge which attains much higher centrifugal force than the ordinary laboratory centrifuge It was invented by Svedberg and a full account of it may be found in the book by Svedberg and Pedersen (19) It consists essentially of a small turbine driven by oil or compressed air or an electric motor which in turn drives a metal chamber called the rotor in which there is a place to put a cell containing the protein solution to be studied This cell has quartz windows so that the sedimentation of the protein under the influence of the centrifugal field may be observed visually or photographically By use of the ultracentrifuge an intense gravitational field may be produced varying from several thousand to as much as a million times gravity In these intense gravitational fields, protein molecules which otherwise would be kept in solution by forces of diffusion are caused to sediment because the forces of diffusion are no longer able to counteract the tendency of the molecules to move away from the center of rotation If a colored protein such as hemoglobin

is employed, visible light may be used to record the sedimentation of the protein A boundary is formed between clear solvent and the protein solution beyond it, and the movement of this boundary can be followed by successive photographs taken at known intervals Knowing the speed of the centrifuge and its dimensions, the centrifugal field may be calculated and thus the *sedimentation constant* of the protein becomes known If the protein were spherical, it would be possible from this alone to calculate its molecular weight Since most protein molecules are not spherical, however, it becomes necessary to know one other thing, the *diffusion constant*, in order that the molecular weight may be determined However, even if this is not known,

TABLE 1

Molecular weights of certain proteins from ultracentrifuge studies

PROTEIN	SEDIMENTATION CONSTANT S_{20}	PARTIAL SPECIFIC VOLUME V_1	FRICTIONAL RATIO f/f_0	MOLECULAR WEIGHT	
				Assuming spherical shape	Corrected for shape
Bacillus phlei protein	1 8	0 748	1 22	13 000	17,000
Tubercle bacillus (human) protein	3 3	0 70	1 25	23 000	32 000
Human serum albumin	4 6	0 733	1 28	47 000	69 000
Diphtheria toxin	4 6	0 736	1 30	49 000	72 000
Rabbit antibody	*7 0*			*95 000*	*157 000*
Human serum gamma globulin	7 2	0 739	1 38	96 000	156 000
Urease (jack bean)	18 6	0 73	1 19	370 000	480 000
Thyroglobulin (pig)	19 2	0 72	1 43	370 000	630 000
Antipneumococcus antibody (horse)	19 3	0 715	1 86	360 000	910 000

a calculation of the molecular weight on the assumption that the molecule is spherical gives a minimal value for the molecular weight and we know that the true molecular weight can not be below this figure It may, however be considerably above it Table 1 shows some typical results for important proteins

If the protein is colorless, as so many are, visible light will not enable sedimentation to be followed by direct observation *Instead it is necessary* to use ultraviolet light which proteins absorb Or by taking advantage of the fact that the refractive index of the protein solution and the solvent will be different, suitable optical equipment makes it possible to photograph *with visible light* the boundary and obtain a photograph in which the boundary is represented by a peak, and the sharper the boundary the sharper and more abrupt will be the peak The student should consult some

other source, such as Svedberg and Pedersen (19), for details of operation and theory Svedberg's high speed ultracentrifuge is driven by a turbine fed by oil under pressure, and the rotor runs in an atmosphere of hydrogen at a pressure of a few millimeters of mercury The hydrogen is necessary to conduct away heat which develops in the rotor during the run A somewhat simpler type to centrifuge driven by compressed air has been developed by various workers in this country A blast of compressed air turns the air turbine which forms the upper portion of the instrument from which the rotor is suspended by a piece of piano wire The piano wire is completely surrounded by a packing which fits very closely and just leaves it free to turn, the very small gap between the wire and the casing being filled by oil The rotor is completely encased and runs in a high vacuum so that the heating effects are extremely small This type of ultracentrifuge is useful not only for molecular weight determinations but for the concentration of viruses and other very large protein molecules, and in general in protein preparations when large amounts are not required The maximum centrifugal force, and thus the resolving power, which can be obtained with this type of instrument is somewhat less than with Svedberg s oil centrifuge but it is satisfactory for all except the smallest protein molecules and is less expensive to construct and operate

Electrophoresis. Another method of separating and characterizing proteins depends upon their characteristic mobility in electric fields Since proteins are amphoteric and can behave either as weak acids or weak bases, they can form salts with strong acids or strong bases and these salts are very highly dissociated—dissociating into a charged protein anion or cation and a metallic cation or anion as the case may be The farther from the isoelectric point we bring the pH, the larger will be the charge on the protein molecule, for then more charged groups will be present Since the isoelectric points of most proteins are somewhat on the acid side of neutrality, it is customary to use an alkaline buffer Plasma proteins are ordinarily run at at a pH of about 8 The protein solution is placed in a U shaped tube of square cross section which is kept at a constant temperature by a thermostated bath Low temperatures are used, not far from the temperature of the maximum density of water, for here the disturbing effects of heating due to passage of electric current are least It can be shown theoretically and it is found experimentally, that once a boundary between solution and proteins has been formed, the electric current will tend to maintain this boundary even though the protein molecules are in motion and subjected to the random forces of Brownian motion On the alkaline side of the isoelectric point the protein ion has a negative charge, consequently it tends to move towards the positive pole of the apparatus An electric potential of the order of 10 volts per centimeter is ordinarily used A schematic diagram

of the apparatus is shown in figure 2b, which gives some idea of the apparatus as developed by Tiselius The boundary between solvent and protein solution is formed mechanically as indicated and the movement of the boundary between solvent and protein solution is followed optically by a method very similar to that used in following the boundary in the ultracentrifuge It is unnecessary to give technical details here, but, again, photographs are obtained which show a peak corresponding to the boundary If more than one protein with different electrophoretic mobilities is present, more than one peak will be obtained, since the molecules will migrate at a different

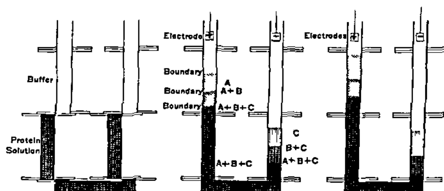


FIG 2b Separation of proteins in electrophoresis At left a solution of three different proteins is placed in the apparatus Buffer is placed in the upper tubes, and the lower tubes containing protein solution slid into contact, giving an uninterrupted path for the electric current (right) and making a sharp boundary In the two diagrams at the right protein A moves fastest protein C slowest The various boundaries when photographed, yield characteristic peaks (figure 3) corresponding to variations in refractive index of the solutions

rate (The rate of movement is practically independent of the size of molecule) In that case more than one peak may be obtained A characteristic photograph is shown in figure 3 which shows results obtained with human plasma Several components will be recognized The designation "A" represents serum albumin, the Greek letter φ stands for fibrinogen, and three species of globulins are found designated by the Greek letters, α, β, and γ The gamma globulins are probably a mixture of proteins of slightly different electrophoretic mobilities More will be said about this when we discuss the components of the blood

There seems to be no simple exact correspondence between protein fractions as identified by electrophoresis and those obtained by salting out procedures However, Cohn *et al* (5) and Svensson (19a) reported a general parallelism between solubility and electrophoretic mobility, so that if rela

tively low salt concentrations were used, the slower moving component (gamma globulin) was precipitated before the faster components. Working with serum Popper *et al.* (17) found that using Wolfson's technique a good correlation between the fractions obtained by salting out and by electrophoresis except with the alpha and beta globulins, was achieved.

Isoelectric point. The isoelectric point of a protein has been defined as a pH value such that the net charge[2] on the amphoteric molecule is zero and it will not move towards either the positive or the negative electric pole. We shall not bother here to distinguish this from the isoionic point, which can be slightly different if the protein combines with anything other than pro-

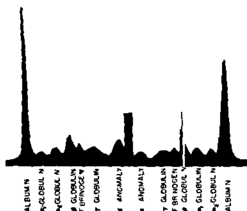


FIG. 3. Electrophoretic schlieren (shadow) diagrams of normal human plasma (courtesy of Dr. J. L. Oncley). Note that in this instance the symbol φ is not used for the fibrinogen.

tons. The isoelectric points of a few typical proteins are given in table 2. It will be observed that a good many proteins have isoelectric points very near together so that this method alone would not distinguish these various proteins very well.

Since a good many aspects of the behavior of proteins are due to their possession of electrical charges, it is not surprising that many of these effects are larger when the protein is not at its isoelectric point and are at a minimum at or near the isoelectric point. Such properties are osmotic pressure, electrophoretic mobility, and swelling of proteins in the solid phase.

The importance of the isoelectric point may be illustrated by the fact that if the pH of a protein is greater than the isoelectric point, it can only combine with cations, forming as a rule a metal proteinate salt, whereas if the pH

[2] Net charge means the algebraic sum of all charges, positive and negative, on the molecule.

is less than the isoelectric point, the protein combines with anions forming a salt of protein and some ions such as chloride ions and so forth This was proved thirty years ago by Jacques Loeb by experiments on gelatin Gelatin was brought to different pH by treatment with varied amounts of nitric acid After washing, the samples were all treated with a solution of silver nitrate of a certain concentration After being washed again, the various samples of gelatin were put in test tubes, melted, and exposed to the light

TABLE 2

Isoelectric points of proteins

PROTEIN	SOURCE	ISOELECTRIC POINT
Actomyosin	Muscle	6 2–6 6
Bushy stunt virus	Infected tomato plant	4 11
Casein	Cow's milk	4 6
Catalase	Beef liver	5 7
Chymotrypsin	Beef pancreas	5 4
Cytochrome C	Beef heart	9 7
Fibrinogen	Human blood	ca 5
Gamma globulin	Human blood	ca 7
Gelatin	Skin	4 8–4 85
Hemoglobin	Horse blood	6 79–6 83
Insulin	Pig's pancreas	5 30–5 35
β Lactoglobulin	Cow's milk	5 18
Myogen	Muscle	6 2–6 4
Old yellow enzyme	Yeast	5 2
Ovalbumin	Hen's egg	4 84–4 90
Papain	Papaya	9
Pepsin	Pig's stomach	2 75–3 0
Salmine	*Salmon sperm*	12 0–12 4
Serum albumin	Human blood	4 9
Trypsin	Beef pancreas	7–8
Urease	*Jack bean*	5 0–5 1

at room temperature, after the pH of the sample had been determined on a sample from each tube All of the gelatin solutions with pH greater than 4 7, which is the isoelectric point of gelatin, became opaque and then brown or black due to the reduction of the protein-silver salt to metallic silver (as in a photographic film), while the solutions with a pH less than 4 7 remained transparent even when exposed to light for months or years

On the acid side of the isoelectric point, that is, when the pH is less than 4 7, gelatin is in combination with the anion of the salt used Loeb demonstrated this by bringing different samples of powdered gelatin to different pH, then treating them for one hour with a dilute solution of potassium

ferrocyanide After this treatment the gelatin was washed thoroughly with cold water and 1 per cent solutions of these different samples made by dissolving in hot water, whereupon it was found that when the pH was less than 4 7 the gelatin solution turned blue after a few days due to the formation of ferric ferrocyanide, some of the iron present having been oxidized to the ferric state All the solutions of gelatin with a pH of 4 7 or above remained permanently colorless

HYDROGEN ION CONCENTRATION AND THE MEASUREMENT OF pH

For some pages now we have been mentioning the symbol pH and have referred to a later definition and discussion of it It now seems time to present this definition, although it is to be hoped that most readers of this book will previously have learned the concepts of pH and know, at least in outline, how it is measured Such readers may omit this section and proceed with the discussion of proteins

The symbol pH was invented by Sørensen as a more convenient way of expressing hydrogen ion concentration As the reader is doubtless aware, the hydrogen ion concentration is of great importance to biological systems, and if it deviates much from neutrality—that is from a hydrogen ion concentration of 10^{-7}—most biological functions are seriously disturbed The reader will recall that the dissociation constant of water times its concentration ($K_{H_2O}[H_2O]$) is equal to 10^{-14} and when hydrogen and hydroxyl ion concentration are equal, or in other words the solution is neutral, the hydrogen ion equals the hydroxyl ion concentration so that $[H^+] = [OH^-]$ and therefore $[H^+]^2 = 10^{-14}$, and $[H^+] = 10^{-7}$ (The brackets indicate concentrations as mols per liter) If the hydrogen ion concentration is ten times as great as that of neutrality, we have a hydrogen ion concentration of 10^{-6} If it is one hundred times as great, the concentration is 10^{-5} And, on the other hand, if the concentration is one tenth that of neutrality, we have hydrogen ion concentration of 10^{-8} The 10 and the minus sign are constant features of all these expressions and Sørensen suggested leaving them out, since they will be understood to be there, and writing only the exponents which he designated by the symbol pH Thus we can see that we may define pH as the negative logarithm of the hydrogen ion concentration The symbol pH has the slight disadvantage that when numbers become larger they designate *lower hydrogen ion concentration or lower actual acidity, and conversely*, smaller numbers designate higher acidity This defect is not serious however and workers with biological systems quickly learn to think in terms of the new symbol

The hydrogen ion concentration, or actual acidity of a solution which we symbolize by pH, should be carefully distinguished from the titratable acidity

which we would obtain by titrating the solution with standard NaOH solution The latter may be much greater in the case of systems containing weak acids, for such acids are not fully dissociated into hydrogen ions (protons) and anions (or in terms of modern physical chemistry, the activity of the hydrogen *ions is much less than that of a strong acid in* the same concentration) As a weak acid is titrated, and H^+ ions are combined with OH^- ions to form water, more H^+ ions are released from the acid, until ultimately all the dissociable hydrogen of the acid has come into play

The way in which hydrogen ion concentration may be measured can be understood from rather elementary electrochemical considerations If a strip of copper is immersed in water or salt solution, a small amount of the copper dissolves and some copper ions are produced until the concentration of copper ion is sufficient to counteract the dissolving tendency of the copper At this point we have equilibrium, and as many copper ions come out of solution and deposit on the copper as go in solution in any given time In other words, we have a dynamic equilibrium

If a copper strip is immersed in a solution containing Cu^{++}, such as a solution of copper sulfate there will be a tendency for copper ions to deposit on the copper strip, forming, when discharged metallic copper Each ion which does so, however, leaves two positive charges on the metallic strip (or takes away two electrons), and before long the charge on the metal is *sufficient to repel the Cu^{++} ions and prevent further deposition of ions so* that the action ceases Suppose, however we have two solutions of copper sulfate at different concentrations and in each of these we immerse a copper strip If we connect the two pieces of copper by an electrical conductor such as a piece of wire, and connect the two solutions by a tube containing ions so as *to permit ion transfer from one solution to the other,* we *will observe* the following action in the more concentrated solution copper is deposited [illegible]

tion current *flows from the positive to the negative pole,* we shall have an electric current flowing from the positively charged strip to that with the negative charge However, since the convention of positive and negative which was adopted at the suggestion of Benjamin Franklin proves to have been just the reverse of the truth, it is unfortunately true that the flow of electrons is in the *opposite direction—that is, from the negative to the posi*tive pole This, however, need not confuse us if we always speak of the electric current as passing from the positive to the negative pole This flow of current will continue with the above set up until the concentration of copper ion has become equal in the two halves of the cell An electric cell of this

type is called a concentration cell because the electric potential produced is due to differences in concentration of the same substance

It is not necessary that the cell be made up of solutions of the same material, however, in fact it is commoner to use cells which are made up of different material This can be illustrated by the following principle If a copper wire is dipped in a tube of water or salt solution some copper ion will dissolve until the back pressure of copper ion tending to deposit out equals the tendency of the copper to dissolve and equilibrium is attained Similarly, if a strip of zinc is dipped in a test tube of water or salt solution, some zinc ions will dissolve but somewhat more since zinc is more soluble than copper in aqueous solution If now we connect the two strips of zinc copper by conducting materials such as a piece of wire and connect the two solutions by glass tubing containing salt solution so that ions may pass from one to the other, we will find that an electric current flows through the wire from the copper to the zinc, in other words, in this cell the copper is the positive pole

At a certain stage of telegraphy not too remote to be remembered by two of the authors of the present book this principle was made use of in the type of battery constructed somewhat as follows in a glass jar was placed a copper electrode covered with a layer of copper sulfate solution On top of this was a layer of zinc sulfate solution of somewhat lower density so it did not sink into the copper sulfate solution Suspended in this solution was a zinc electrode The two were connected by a wire and a current was found to flow from the copper to the zinc (A salt bridge was not necessary in this case as the two solutions were in direct contact) The zinc was gradually eaten away while copper deposited out of solution onto the copper electrode As long as the battery was kept connected the electric action itself prevented the two solutions from mixing by diffusion So called dry cells containing similar materials in the form of pastes are still in use today for flashlights and electric bells

If we now go back to the concentration cell, we see that it seems reasonable to expect that there will be a relationship between the concentration of ions in the two sides of the cell and the electromotive force developed between the electrodes This is in fact true and the equation for the electromotive force is well known This means that if we knew the concentration of ions in one side of the cell and measured the electromotive force, we could calculate from the equation what the concentration of ions was in the other side of the cell On this principle is based the determination of hydrogen ions

To determine hydrogen ions one would want a set up which would contain two cells one with a known hydrogen ion concentration, the other

with an unknown hydrogen ion concentration which we desired to determine If we could immerse electrodes of solid hydrogen in these and get electrical action when the two electrodes were connected by a wire and the solutions connected by a salt bridge such measurements could be made Unfortunately the freezing point of hydrogen is far too low for us to make use of such a simple device not to mention other difficulties However it has been found possible to use *gaseous* hydrogen, if it is kept in the small pores and crevices on the surface of some non reactive conducting surface One of the so called noble metals such as platinum gold or palladium is satisfactory for this purpose Then if we have an electrode of such material and keep the pores filled with gaseous hydrogen by bubbling hydrogen over the electrodes constantly we have what is the equivalent of a hydrogen electrode If we put a known hydrogen ion concentration on one side then we measure the ion concentration in an unknown solution on the other side of the cell

In practice it has not proved convenient to make up known hydrogen ion concentrations for use in such determinations because they do not remain constant in concentrations but tend to change by evaporation and other effects But from what has been said above about the copper zinc cell it will be apparent that if we replace the hydrogen half cell on one side of the hydrogen cell just described by some other half cell we will still get an electric potential and it is known that this will differ by a constant from the potential which we would have obtained with a hydrogen electrode of known hydrogen ion concentration The so called calomel electrode has been found most satisfactory for this purpose

We now see that if we have a calomel half cell or calomel electrode as it is usually called and a half cell containing a hydrogen electrode and a hydrogen ion concentration which we wish to determine we may do so by connecting the two by a suitable salt bridge and measuring the electromotive force between the two electrodes with a potentiometer When this is done we may calculate from an equation the hydrogen ion concentration in the unknown *solution*

The hydrogen electrode as just described is the basis of all accurate [illegible]

hydrogen electrode by a so called glass electrode [illegible] of a thin glass membrane which is brought in contact on the outside with the unknown solution and on the inside with a solution of fixed hydrogen

ion concentration, so arranged that this known H^+ concentration can be sealed in and thus will not change Such a glass electrode in connection with a calomel electrode together form a cell capable of measuring hydrogen ion concentration It is possible to calibrate the instrument so that it will read directly in pH units These are the most accurate and simplest of the common laboratory instruments for the measurement

A brief mention should be made of other methods of determining hydrogen ions Many chemical compounds change color at certain pH (hydrogen ion concentrations)—one color when alkaline to this pH and another color,

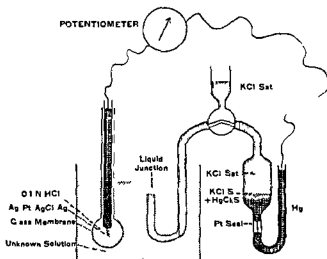


FIG 4 Diagram of glass electrode pH meter Glass electrode at left calomel half cell at right The potentiometer consists of a galvanometer plus batteries a Wheatstone bridge circuit and a vacuum tube amplifying circuit not shown here

or colorless, when acid to this pH Such substances are called indicators and their characteristic color change may be made use of to determine pH During the range in which part of the indicator is in one colored form and part in a form having a different color (or colorless), a blend of color results, giving a range of different shades or grades of color By comparing these with the color obtained in solutions of known pH, the pH of an unknown solution may be determined The best known indicator is probably litmus, which can be deposited on paper and every student of biochemistry will have used litmus paper in his early study of chemistry Indicators are now known which cover the whole range of pH which we desire to investigate Their results are not as accurate as those of the glass electrode, but they are quicker to use and often convenient

with an unknown hydrogen ion concentration which we desired to determine. If we could immerse electrodes of solid hydrogen in these and get electrical action when the two electrodes were connected by a wire and the solutions connected by a salt bridge, such measurements could be made. Unfortunately, the freezing point of hydrogen is far too low for us to make use of such a simple device, not to mention other difficulties. However, it has been found possible to use gaseous hydrogen, if it is kept in the small pores and crevices on the surface of some non reactive conducting surface. One of the so-called noble metals such as platinum, gold, or palladium is satisfactory for this purpose. Then if we have an electrode of such material and keep the pores filled with gaseous hydrogen by bubbling hydrogen over the electrodes constantly, we have what is the equivalent of a hydrogen electrode. If we put a known hydrogen ion concentration on one side then we measure the ion concentration in an unknown solution on the other side of the cell.

In practice it has not proved convenient to make up known hydrogen ion concentrations for use in such determinations, because they do not remain constant in concentrations, but tend to change by evaporation and other effects. But from what has been said above about the copper zinc cell it will be apparent that if we replace the hydrogen half cell on one side of the hydrogen cell just described by some other half cell, we will still get an electric potential and it is known that this will differ by a constant from the potential which we would have obtained with a hydrogen electrode of known hydrogen ion concentration. The so called calomel electrode has been found most satisfactory for this purpose.

We now see that if we have a calomel half cell, or calomel electrode as it is usually called, and a half cell containing a hydrogen electrode and a hydrogen ion concentration which we wish to determine, we may do so by connecting the two by a suitable salt bridge and measuring the electromotive force between the two electrodes with a potentiometer. When this is done we may calculate from an equation the hydrogen ion concentration in the unknown solution.

The hydrogen electrode as just described is the basis of all accurate hydrogen ion determinations. For practical purposes, however, it has been superseded by other more convenient types of apparatus. For example, it was discovered that if a thin glass membrane was brought into contact with a solution containing hydrogen ions, a potential was developed across ... the

hydrogen electrode by a so called glass electrode ... of a thin glass membrane which is brought in contact on the outside with the unknown solution and on the inside with a solution of fixed hydrogen

mechanisms for these two phenomena are entirely different Gas pressure is due to the impact of the molecules on the walls of the container, osmotic pressure is due to the decrease of thermodynamic activity of the solvent due to the presence of the solute Also, in the thermodynamic derivation of the expression for osmotic pressure, it will be found that the way in which V, R, and T come into the expression is quite different from that in the gas law

The osmotic pressure of a solution depends on the number of particles of solute contained in unit volume From this it follows that if one gram of protein is dissolved in 100 ml of water, the osmotic pressure will be much less than that produced by dissolving one gram of sugar and this in turn is less than that produced by dissolving one gram of sodium chloride—the reason being of course, that the number of particles is larger the smaller the molecular weight and also, in the case of sodium chloride, dissociation increases the number still further

Osmotic pressures of protein solutions are more difficult to measure than might be supposed because (a) the osmotic pressures are fairly small because of the large size of the molecules and it is difficult to obtain readings with high percentage accuracy, the error being approximately constant, the proportional error will vary inversely with the size of the total reading (b) In early work methods were employed which required a long time to obtain equilibrium, allowing alteration or decomposition of the protein to take place (c) If the protein is not isoelectric, but combined with anions or cations, these ions increase the observed value of the osmotic pressure because of the Donnan equilibria which are set up (The Donnan equilibrium is discussed later) (d) The measurement is only an estimate of the mean molecular weights of the non diffusible components of the system It gives no indication as to whether the protein preparation is monodisperse (all molecules the same size) or whether it is a mixture of proteins of different sizes

It is not surprising therefore that the first really good measurements of molecular weights of proteins by osmotic pressure were not done until 1925 and that these results were confirmed by the then new ultracentrifuge within one year When the early ultracentrifugal results were reported there was some skepticism, particularly in regard to those molecular weights which ran into the millions, but this skepticism was dispelled by osmotic pressure measurements on hemocyanin solutions which, although they did not give the correct values because of the very large proportional error involved in measuring the osmotic pressure of these very large molecules, did show that the molecular weight was of the order of one or several million

Sedimentation constant. The ultracentrifuge has been discussed previ

solution, under suitable conditions The osmotic pressure may be defined as the hydrostatic pressure which would be applied to the solution to produce equilibrium if the solution is separated from pure solvent by a membrane which is freely permeable to the solvent but impermeable to the solute In the case of proteins, because of their large molecular size, such membranes are not very difficult to procure Note that the pressure which we have defined as the osmotic pressure has to be exerted *on* the solution The reason for this is that when the solution is in contact with the solvent through this membrane, solvent tends to pass more rapidly in the direction from solvent to solution than in the opposite direction, so that solvent is gradually drawn into the solution To counteract this, pressure must be exerted on the solution until this counterpressure is sufficient to counteract the tendency of the solvent to move from one part of the membrane to the other It is only in this sense that osmotic pressure is a pressure, otherwise it would be more accurate to describe it as a kind of suction, for it is the solution which sucks water into itself The reason *osmotic pressure is important biologically is because solutions have the* power of drawing water into themselves through a semipermeable membrane The body contains many such membranes

If we represent the osmotic pressure by the Greek letter π, its value is given *for dilute solutions by the expression*

$$\pi = \frac{RTx}{V_o}$$

where R is the gas constant, T the absolute temperature, x the mol fraction of the solute, and V_o the volume occupied by one mol of solvent Or, clearing of fractions, we get

$$\pi V_o = xRT$$

Another source of confusion is the emphasis which has been laid in the past upon the formal similarity between these equations for osmotic pressure and the perfect gas law [3] It is true that in very dilute solutions the osmotic pressure is the same as the pressure which would be exerted at the same temperatures by gas molecules in the same volume concentration as the solute molecules There, however, the similarity ends The

[3] The perfect gas law is an expression giving the behavior of a perfect gas ', as idealized from the behavior of actual gases, all of which are "imperfect", although not always in the same way It is given by

$$PV = NRT$$

where N is the number of mols of gas in the container and the other symbols have the same significance as above The perfect gas law is a law approached as a limit by actual gases as the pressures studied become less and less

proton is removed from a cationic acid group, both the net charge and the total charge decrease by one unit Thus, if the protein contains +n cationic acid groups the net charge and also the total charge in strongly acid solutions is +n When protons are removed from the protein ions by the addition of base, the net charge on the protein molecule falls from +n to zero, although the total charge on the protein in this condition may be, in fact usually is, greater than on the cationic protein After the protons have been removed by the addition of base, the pH reached is called the isoionic point If only protons are involved, this may correspond to the isoelectric point of the protein as determined by electrophoresis In this book we shall make no distinction between the two For a definition of cationic acids, see Appendix IV

It is clear that the increase in net charge on the protein molecule between the isoionic point and the region of maximum acid binding capacity equals the number n—that is, the number of cationic groups in the protein By similar reasoning it is clear that the maximum base binding capacity of the protein should be equal to the number of uncharged acid groups of molecules Thus, from titration curves of the protein we are able to draw some conclusions as to the number of basic and acidic groups present in the molecule The titration curve of human serum albumin is shown in figure 5 It will be noted that the curve shows two steep drops The first, and steepest, is roughly between pH values of 3 5 and 4 5 The second is roughly from pH 9 to 12 It is probable that in the first region the carboxy groups of the dicarboxylic amino acids are being titrated, and in the second the amino or other basic groups, lysine and arginine (or hydroxyl groups of tyrosine) residues In the flatter region in between, the histidine groups probably come into play (3)

Immunological Characterization

When a foreign protein is injected into an animal, the animal frequently responds by the production of substances capable of combining with this protein, sometimes in a visible way so as to produce a precipitate The substances produced in response to the injection are called antibodies and they are relatively specific for the protein in response to which they are produced For instance, if we inject the albumin from the hen's egg into a rabbit, we usually obtain antibodies capable of precipitating the albumin from the hen's egg [illegible]

is not complete but tends to follow taxonomic lines, in other words, when species are closely related, their corresponding proteins are likely to be similar immunologically The immunological method affords the best way

ously and it was pointed out that from the sedimentation constant a value of the molecular weight could be deduced if it were shown that the molecule were spherical If the molecule is not spherical it is necessary to know the diffusion constant of the protein and diffusion constants are in general *known less accurately than sedimentation* constants Therefore it is customary to give the sedimentation constant of a protein as one of its characteristics without attempting an estimated molecular weight unless a reliable value of the diffusion constant is available The sedimentation constant is now expressed in units of svedbergs named after the inventor of the ultracentrifuge Values of sedimentation constant, usually symbolized S_{20} of the order of 2, 3 3 5 and so on, have been observed up to a value of 280 for a *virus preparation This latter preparation* was *considered to* have a molecular weight about 47 million

Light-scattering methods When light is passed through a protein solution some of it is scattered in various directions by the molecules of proteins Quantitative measurements of this phenomenon enable us to estimate (a) the size of the protein molecules This depends upon the fact that the apparent turbidity varies with the wave length of the light used *and also depends on the size of the molecules The dependence of the* turbidity on the wave length has been shown to make an estimate of the particle size or molecular weight possible (b) The way in which the light is scattered in different directions has been shown to depend on the shape of the particles and a study of this with light of different wave lengths enables the shape of the particles to be calculated

Light scattering methods are coming into increasing use in protein research and may eventually become one of the regular procedures in protein chemistry These methods have the advantage of being relatively rapid

Titration Curves

The amino acids of which proteins are composed contain several different types of acid and basic groups Therefore one characteristic of any given protein is the amount of acid or base which must be added to bring it from one given pH to another or if we start at the extreme acid or base range we obtain a curve of the equivalents of acid or base added plotted against p f the protein

If the *that the protein* molecule ition of hydroxyl ion brings about the removal of protons from the acidic group of the protein If the proton is removed from an uncharged acid group, a negatively charged group is produced Thus the total charge *on the protein is in*creased by one unit while the net charge is decreased by one unit If a

proton is removed from a cationic acid group both the net charge and the total charge decrease by one unit Thus if the protein contains +n cationic acid groups the net charge and also the total charge in strongly acid solutions is +n When protons are removed from the protein ions by the addition of base the net charge on the protein molecule falls from +n to zero although the total charge on the protein in this condition may be in fact usually is greater than on the cationic protein After the protons have been removed by the addition of base, the pH reached is called the isoionic point If only protons are involved this may correspond to the isoelectric point of the protein as determined by electrophoresis In this book we shall make no distinction between the two For a definition of cationic acids see Appendix IV

It is clear that the increase in net charge on the protein molecule between the isoionic point and the region of maximum acid binding capacity equals the number n—that is the number of cationic groups in the protein By similar reasoning it is clear that the maximum base binding capacity of the protein should be equal to the number of uncharged acid groups of molecules Thus from titration curves of the protein we are able to draw some conclusions as to the number of basic and acidic groups present in the molecule The titration curve of human serum albumin is shown in figure 5 It will be noted that the curve shows two steep drops The first and steepest is roughly between pH values of 3 5 and 4 5 The second is roughly from pH 9 to 12 It is probable that in the first region the carboxy groups of the dicarboxylic amino acids are being titrated and in the second the amino or other basic groups lysine and arginine (or hydroxyl groups of tyrosine) residues In the flatter region in between the histidine groups probably come into play (3)

Immunological Characterization

When a foreign protein is injected into an animal the animal frequently responds by the production of substances capable of combining with this protein sometimes in a visible way so as to produce a precipitate The substances produced in response to the injection are called antibodies and they are relatively specific for the protein in response to which they are produced For instance if we inject the albumin from the hen s egg into a rabbit we usually obtain antibodies capable of precipitating the albumin from the hen s egg This will not react with most other proteins of the egg or chicken tissues but will react although somewhat less satisfactorily with the protein from the egg of the duck and other birds The specificity is not complete but tends to follow taxonomic lines in other words when species are closely related their corresponding proteins are likely to be similar immunologically The immunological method affords the best way

of characterizing proteins sharply and enables us to distinguish proteins which may seem identical from the point of view of molecular weight, electrophoretic mobility, and so forth By quantitative studies of this pre-

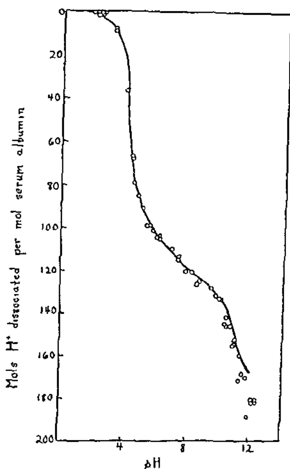


FIG 5 Titration curve of normal human serum albumin (Tanford) Mols of hydrogen ion dissociated per mol of serum albumin plotted against pH

cipitation reaction it is possible to estimate the degree to which the proteins are related to each other

However, these immunochemical, or serological methods as they are sometimes called, have certain drawbacks as methods of characterizing proteins Individual animals *do not always respond in the same way to the* injection of foreign proteins, and the antibodies produced by one animal may indicate more similarity between proteins than do the antibodies pro

duced by another Also, the antibodies do not keep indefinitely after the animal has been bled so that the antiserum is not a reagent of constant properties—this introduces some complications into its routine use to characterize and identify proteins However the method has had valuable applications

More will be said about immunological reactions in the chapter on infection

Colloidal Behavior of Proteins

The distinction between crystalloids and colloids was proposed by Graham in 1861, crystalloids were characterized by a tendency to form crystals when separating from watery solutions and colloids by a tendency to separate out in the form of gelatinous or amorphous masses Graham found that these two groups of substances differed also in two other respects first, in their diffusive mobility, and second, in their peculiar physical aggregation The crystalloids diffuse readily through various membranes for example, the wall of the pig's bladder but the colloids are able to diffuse not at all or only very slowly through these membranes The second peculiarity was the tendency of the colloids to form aggregates when in solution, while this property was lacking or less pronounced in crystalloids

Later workers came to the conclusion that there was no such fundamental distinction between colloidal and crystalloidal substances as Graham had *thought, and distinguished instead between colloidal and crystalloidal* states of matter For instance, Krafft observed that the alkaline salts of the higher fatty acids, stearate, palmitate, oleate, dissolve in alcohol as crystalloids of normal molecular weight, but in water they are true colloids The reverse is true of sodium chloride In water, of course, sodium chloride is a typical crystalloid but Paal found that the latter gave a colloidal solution in benzene

Later, Loeb showed that it was possible to explain practically all of the characteristics of colloidal behavior merely by the difference in diffusibility between colloids and crystalloids The tendency to form aggregates is of slight importance in explaining the colloidal behavior of the proteins, although some protein molecules will aggregate under suitable conditions It is also true that the protein molecule as we ordinarily know it can often be dissociated into smaller units under more extreme conditions But these units are nevertheless of the order of the size of protein molecules and are still colloidal in their behavior Therefore the formation of aggregates is not the secret of the colloidal behavior of proteins, it is their large size their amphoteric properties, and their inability to pass through membranes which allow ordinary electrolytes to pass that give this behavior

Diffusibility of Proteins

Proteins pass either in very small quantities or not at all through membranes such as cellophane which allow electrolytes to pass freely As already mentioned, this difference in diffusibility can be taken advantage of in attempts to purify proteins Also, this non diffusibility of proteins is of great importance to living matter, serving to keep the structural units in their proper place

Osmotic Pressure of Proteins

The osmotic pressure of proteins has already been discussed The ways in which they behave as typical colloids are two (a) a protein exerts a rather small osmotic effect per unit weight This is because of its large molecular weight (b) It is not necessary to have membranes with pores as fine as would be necessary for substances such as sugar in order to measure the osmotic effect of a protein Relatively coarse membranes such as various types of collodion, and more recently cellophane, may be used

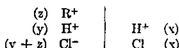
Donnan Membrane Equilibrium

The British chemist, Donnan, proved in 1911 (6) that when a membrane separates two solutions of electrolytes one of which contains an ion which can not diffuse through the membrane while the other ions can diffuse through the membrane, the result will be an unequal distribution of the diffusible ions on the opposite sides of the membrane At equilibrium the products of the concentration of each pair of oppositely charged diffusible ions are the same on the opposite sides of the membrane This unequal concentration of the crystalloidal ions gives rise to potential differences and osmotic forces, and it was shown by Loeb that these forces furnish the explanation for the colloidal behavior of proteins

Donnan derived his results from thermodynamic considerations, and students familiar with these methods may find this derivation either in the original paper (6) or quoted in Loeb's book (13) Actually, Willard Gibbs (7) had derived the general equations for this and similar equilibria, although he did not cite any specific examples Indeed, at the time Gibbs wrote, none were known Modern writers familiar with Gibbs' work often refer to the result as the Gibbs Donnan equilibrium

To derive the Donnan equilibrium let us consider a system of two compartments each containing an aqueous solution, separated by a semipermeable membrane such that all but one of the ions present can penetrate it This non-diffusible ion must perforce remain in the compartment in which it is first placed Water and the diffusible ions are however able to penetrate the membrane and will eventually be distributed, as a result of diffusion, between the two compartments in such a way that we have an equilibrium

If the electrolytes present are hydrochloric acid (HCl) and protein chloride, which we may represent by RCl the membrane being impermeable to ion R^+ then the ions H^+ and Cl^- can penetrate the membrane but they must be considered as going through in pairs, not separately because of the electrostatic attraction which one exerts on the other Electrostatic forces are so great that in a solution no large number of positive charges is ever separated from a large number of negative charges Now the probability that a single ion arrives at a given point of the membrane during a given time interval is obviously proportional to the concentration of that ion in the solution on that side of the membrane The probability that another ion will hit a point on the membrane is proportional to *its* concentration in the solution The probability that two independent events will happen simultaneously is equal to the product of the individual probabilities Therefore, the probability that an H^+ ion and a Cl ion will simultaneously arrive at a given point at the membrane is equal to the product of their concentrations Thus the rate of diffusion of HCl through the membrane in one direction is proportional to the product of the concentrations of the H^+ ion and Cl^- ion in the side from which it is diffusing and the same is true for its diffusion through the membrane from the opposite side At equilibrium the two rates of diffusion must be equal The diagram represents the equilibrium state



In the diagram the vertical line represents the membrane and the chemical symbols the various ions We let the letter x represent the concentration of H^+ (or Cl^-) ions which we remember must be equal because of the electrostatic forces between them Let y represent the concentration of H ions on the other side of the membrane, and z the concentration of R^+ ions Then, $y + z$ = concentration of Cl^- ions on that side of the membrane for here, too electroneutrality must be maintained The Donnan equilibrium which as we have seen implies that the products of the concentrations on the two sides must be equal for the two ions H^+ and Cl^- gives us the relation $x^2 = y(y + z)$ It is easy to see from this equation that x is greater than y, since x^2, y^2 and z^2 are necessarily all positive numbers, and x^2 being the sum of y^2 and yz, is greater than y^2 It is also obvious from the equation that x is equal to the geometric mean between y and $y + z$ Consequently, x must be intermediate in value between y and $y + z$ Therefore we see that, although the ions H^+ and Cl^- can pass freely through the membrane, their concentrations on the two sides at equilibrium will not be equal if a non-diffusible ion is present on one side This un

equal distribution of ions is the outstanding feature of the Donnan equilibrium and accounts for the influence of electrolytes on various properties of colloid systems

One consequence of the difference in concentrations of the diffusible ions is that there will be a potential difference between the opposite sides of the membrane The measurement and significance of such potentials in the body forms a part of biophysics Such potentials are involved in neuromuscular activity and in glandular secretion Further information will be found in textbooks of physiology

For the operation of the Gibbs Donnan membrane effect it is not necessary to have the protein enclosed in a membrane such as collodion or cellophane *If the protein is in the solid state or forms part of a structure such* as a cell which prevents it from moving through the solution, it is just as effectively immobilized as if it were confined within a semipermeable membrane Under such conditions the Gibbs Donnan equilibrium is observed For instance if small particles of gelatin are suspended in cold water or salt solution at various pH, it will be found that the salt concentration and the pH inside and outside differ in accordance with the membrane equilibrium as defined above It is also found that the sign of the charge inside the particles of gelatin depends upon the pH, just as would be predicted The same considerations apply to a suspension of any amphoteric compound capable of combining with the electrolytes of the medium

The Donnan membrane equilibrium also enables us to account for the effect of salts on colloidal systems If we have an acid gelatin solution or if particles of gelatin are suspended in an aqueous acid medium the potential difference between the positive gelatin ions and the solution with which it is in contact is determined by the values in the Donnan equation for the anions The values of the cations do not enter into the expression

expected amounts by adding calculated amounts of various salts He was also able to show that the effect depended on the anions added and was independent of the concentration and valency of the cations Loeb noted that this explained the precipitating effect of the salt on otherwise stable colloidal suspensions and thus corrected an error which had long appeared in the literature of colloidal chemistry, to the effect that neutral salts precipitated colloidal particles by bringing them to their isoelectric point What happens is that the addition of neutral salts diminishes the potential difference between the suspended particles and the liquid, and if enough salt is added it completely wipes out this potential difference It is true

that if the particles, gelatin granules, for example, are brought to their isoelectric point by a change in hydrogen ion concentration the potential difference between the particles and the surrounding liquid also becomes zero, but this is for a different reason, mainly because the net charge on the gelatin molecule is zero at this point

The Gibbs Donnan equilibrium also explains the effect of salts on the osmotic pressure of proteins It has been known for a long time that the osmotic pressure of a protein solution as measured at pH values not at the isoelectric point may be considerably larger than would be expected Errors of considerable magnitude have resulted from making osmotic pressure measurements not at the isoelectric point The measured osmotic pressure of a protein separated from water by a semipermeable membrane is the sum of the pressure due to the colloidal particles and the pressure caused by the difference in the number of dialyzable ions inside and outside the membrane This difference can be calculated and measurements have been made on colloidal systems which agree well with theory The great difficulty of obtaining proteins absolutely free from electrolytes and exactly at their isoelectric points accounts for the inaccuracy of the osmotic pressure measurements and the resulting molecular weight determinations with the larger protein molecules It is for this reason that other methods such as the ultracentrifuge often give more reliable results

Shape of Protein Molecules

It was stated above that there was evidence that most protein molecules are not spherical Measurements of the viscosity of protein solutions indicate that protein molecules are either fairly elongated ellipsoids—something like a cigar—or else flat disks—something like a peppermint wafer The diffusion and viscosity data could be interpreted on either one of these assumptions These calculations are based, however, on the assumption that the protein molecule is not hydrated—in other words that it does not bind any water but simply swims in a sea of solution It is likely that water does combine in a relatively firm way with proteins in solution so that proteins are *hydrated*, and if this is taken into account, the ratio of the major and minor axes of the molecule and the viscosity are related to the hydration The decision between the elongated ellipsoid and the flattened disk as the true shape of protein molecules in solution has to be made by determination of dielectric increment and dispersion and relaxation time Such work has been published by Oncley (3) and he states that the agreement between the various methods for evaluation hydration and the ratio of the two axes yields strong support for the view that protein molecules appear to rotate as rigid units which can be mathematically described

as if they were elongated ellipsoids of revolution with varying ratios of major minor axes In figure 6 we give pictures of the probable appearance of certain well known protein molecules

Denaturation and Coagulation of Proteins

Proteins as we have already stated, are very labile molecules Any change which leaves a protein molecule in a state which we judge to be different from the state in which it is found in those tissues which are its natural source is called denaturation Since we usually have to judge this

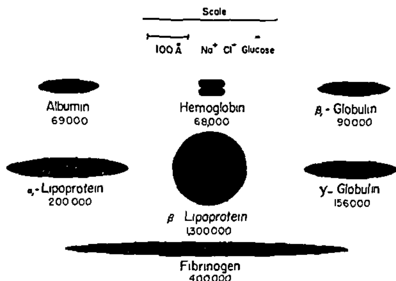


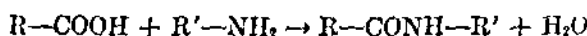
FIG 6 Probable appearance and relative dimensions of certain protein molecules (*courtesy of Dr J I Oncley*)

by comparison with the sample of protein which we had extracted by what we think is a safe procedure, it is not always easy to make certain that a given sample of protein has not been denatured It is easier to state in some cases that it definitely has been denatured One of the first symptoms of denaturation is a loss of solubility, this loss may be partial or complete When the loss is complete the protein is said to be coagulated, it becomes an insoluble solid This may be effected by drastic treatment such as boiling or the action of nitric acid and other powerful reagents Albumins or globulins are coagulated by heating their solutions to the boiling point Some are coagulated at lower temperatures One seems to be reversibly coagulated and redissolves at higher temperatures, this is the Bence-Jones protein which occurs in the urine in certain types of malignancy (see

page 668) But not all proteins are coagulated by heating to the boiling point Casein for instance, may be boiled or even autoclaved, and still remain in solution What happens during denaturation will be discussed in Chapter 2

AMINO ACIDS

There is convincing evidence, which will be presented in the next chapter, that proteins are primarily made up of amino acids which are held together by the *peptide linkage* That is, the carboxyl group of one amino acid has combined with the amino group of another, water is split off, and the —CONH— link is formed

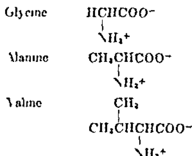


$$R—COOH + R'—NH_2 \rightarrow R—CONH—R' + H_2O$$

All the amino acids naturally occurring in proteins are α amino acids—in other words, have an amino group attached to the carbon next to the carboxyl group Some have also an amino group elsewhere Also certain amino acids have two carboxyl groups It is mainly the presence of these extra amino and carboxyl groups which seem to take no part in the peptide linkage holding the protein together, which explains the amphoteric properties of proteins and their behavior as dipolar ions At suitable pH a free carboxyl group can ionize, giving R—COO plus a proton and under other conditions an amino group can acquire a proton giving us $R—NH_3^+$ Some amino acids contain also other groups capable of ionizing such as hydroxyl, imidazole, and guanidine

The amino acids which have been determined to be natural constituents of proteins may be classified in various ways Following Cohn and Edsall (3) we may divide them into the following classes Each class contains amino acids with a certain type of group which confers characteristic properties on the molecule

1 Monoamino monocarboxy-α amino acids whose side chains differ only in the length of the hydrocarbon chain and its degree of branching

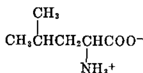


Glycine $HCHCOO^-$ with NH_3^+

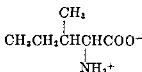
Alanine CH_3CHCOO^- with NH_3^+

Valine CH_3 / $CH_3CHCHCOO^-$ with NH_3^+

Leucine

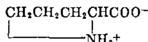


Isoleucine

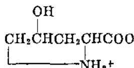


2 Amino acids containing pyrrolidine rings rather than paraffin side chains, and which are imino acids rather than amino acids

Proline

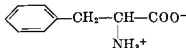


Hydroxyproline

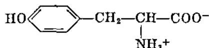


3 Amino acids containing benzene or indole rings in the side chains

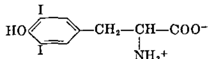
Phenylalanine



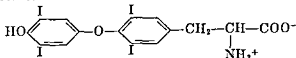
Tyrosine



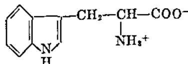
Diiodotyrosine



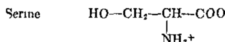
Thyroxine

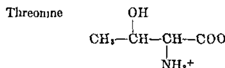


Tryptophane

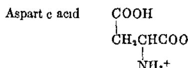


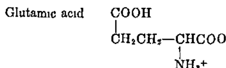
4 Amino acids containing hydroxyl groups whose acidity is so feeble that it makes no contribution to the amphoteric properties of the protein



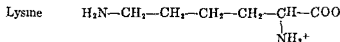


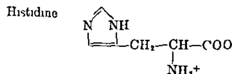
5 The dicarboxy amino acids

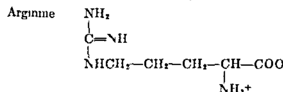




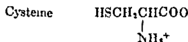
6 Diamino and other basic amino acids







7 The sulfur containing amino acids



Simple Proteins

The simple proteins on hydrolysis yield α amino acids or their derivatives only

1 *Albumins* are soluble in water and are coagulated by heat They are obtained from animal and vegetable cells and body fluids Good examples are egg albumin, serum albumin from blood, and perhaps leucosin from wheat It is interesting that these definitions begin to break down even at the very beginning of the classification, for some of the so called simple proteins—characteristic in other ways—contain other components in addition to amino acids, for instance, crystalline egg albumin even from repeatedly crystallized preparations of the highest purity contains a polysaccharide made up of mannose and glucosamine groups

2 *Globulins*, found in the same sources as the albumins are insoluble in pure water but are soluble in dilute salt solution and are coagulated by heating Like albumins, they are precipitated at higher salt concentrations, somewhat more readily than are albumins The best examples of globulins are perhaps serum globulin, which has been prepared in quite pure although not crystalline form, myosin from muscle, and ovoglobulin from egg yolk Also certain plant proteins are put in this class edestin from hemp seed, amandin from almond, and excelsin from Brazil nuts These proteins, however, require considerably higher salt concentrations, of the order of 5 per cent NaCl, to keep them in solution, and they are more readily crystallized

Sørensen distinguished two kinds of globulins The first kind, euglobulin is insoluble in pure water but soluble in dilute salt solutions It was reported to contain phosphorus The second kind, pseudoglobulin is soluble in pure water and was reported not to contain phosphorus The pseudoglobulins were distinguished from the albumins by being salted out, like other globulins, by half saturation with ammonium sulfate In practice it is hard to distinguish euglobulin from pseudoglobulin largely because of the great difficulty of getting proteins entirely salt free Globulins homogeneous by ultracentrifugal and electrophoretic test, sometimes prove to be partly soluble in pure water and partly insoluble, with no striking difference in other properties between the two fractions

3 The *glutenins*, obtained from cereal seeds, are insoluble in all neutral solvents, but are soluble in dilute acid and dilute alkali Examples are glutenin from wheat and oryzenin from rice

4 Alcohol soluble proteins (*prolamines*), also from seeds, are soluble in 70 to 80 per cent alcohol but insoluble in either water or absolute alcohol Examples are gliadin from wheat, hordein from barley, and zein from Indian corn (*Zea mays*)

5 The *scleroproteins* or *albuminoids* are also insoluble in neutral solvents and practically insoluble in other solvents Examples are collagen from hide, bone, and cartilage, and keratin from skin, horns hair, and feathers These proteins have a high content of the sulfur containing amino acids

6 The *histones*, from animal cells are soluble in water but insoluble in dilute ammonia because of their basic properties Solutions of many other proteins will precipitate with histones Examples are globin, which is the protein part of hemoglobin, thymus histone, and scombrone from mackerel

7 The *protamines* are found in ripe generative cells of some fish and are the simplest of the simple proteins They have a low molecular weight for proteins, they are soluble in water and not coagulated by heating, they precipitate many other proteins from their aqueous solutions They contain relatively few amino acids and these are largely basic in character Therefore the protamines possess strong basic properties and form stable salts with the strong acids Examples of protamines are salmine from salmon, sturine from sturgeon, clupeine from herring, scombrine from mackerel, and cyprinine from carp

Conjugated Proteins

Conjugated proteins are proteins combined with non protein compounds other than salts The non protein portion is called the prosthetic group

1 The *nucleoproteins* are combinations of proteins with nucleic acids Nucleoproteins will be discussed more in detail in Chapter 7

2 The *glycoproteins* are combinations of proteins with substances which contain a carbohydrate group Examples are mucin which occurs in saliva osseomucoid from bone, and tendomucoid from tendons

3 The *phosphoproteins* are combinations of proteins with phosphorus containing substances other than nucleic acid or phospholipid Examples are casein from milk, and possibly vitellin from egg yolk

4 The *chromoproteins* are combinations of protein with a colored prosthetic group The best known example is hemoglobin Copper containing chromoproteins called hemocyanins occur in the blood of a number of invertebrates such as lobsters and crabs

5 The *lipoproteins* are combinations of proteins with lipids They are found in cell nuclei, egg yolk, milk, and in the blood They are widely distributed in blood and tissues, in some viruses, and in certain bacterial antigens

Derived Proteins

As the name implies derived proteins are proteins which have been derived from other proteins by modification of one sort or another They are divided into 1) primary protein derivatives, which include proteans,

metaproteins, and coagulated proteins, and 2) secondary protein derivatives, which include proteoses peptones, and peptides The first group has been changed relatively slightly from the original protein the second group has been more extensively changed, and in the case of peptides has been degraded to relatively small molecules, in many cases so small that they can be identified as definite chemical compounds of known composition The best known derived protein, gelatin, is difficult to put snugly into either of these classes It is prepared by the treatment of collagens (from bone and so forth) with superheated steam or boiling dilute acids, and is certainly radically altered from its parent proteins In regard to size it is intermediate, consisting of a mixture of molecules of various sizes, from molecular weights of the order of the albumins down to relatively small molecules The average molecular weight is so small that much of an ordinary gelatin preparation can pass through membranes such as the capillary walls Another notable feature of gelatin is its non antigenicity

1 The *proteans* are insoluble products which result from the action for a short time of various reagents, such as dilute acids, or enzymes, or even water Examples are myosan from myosin, and edestan from edestin

2 The *metaproteins* are the result of still further action of acids and alkalis They are soluble in dilute acids and alkalis but insoluble in solutions of neutral salts Examples are acid albuminate and alkali albuminate

3 The *coagulated proteins* are insoluble products resulting either from the action of heat or other denaturing agents, such as alcohol or formaldehyde

4 The *proteoses* are soluble in water and are not coagulated by heat They can be precipitated if their solutions are saturated with ammonium sulfate or zinc sulfate, typical protein precipitants

5 The *peptones are soluble in water and not coagulated by heat* They are not precipitated even if their solutions are saturated with ammonium sulfate This is partly due to their lower molecular weight Certain reagents which are known to precipitate alkaloids also precipitate peptones An example is phosphotungstic acid

6 The *peptides* are combinations of two or more amino acids joined together by the peptide link Many of them have been definitely identified chemically An interesting example is glutathione, which is γ glutamyl cysteinylglycine

REACTIONS OF PROTEINS, ESPECIALLY COLOR REACTIONS

In our original definition of proteins we mentioned that they were made up chiefly of α amino acids and that the separation of α amino acids from the products of hydrolysis of a substance would establish its protein nature Since this is difficult and time-consuming, it is convenient to have various color tests which indicate that a protein, or some other substance which

happens to give the test, is present If several of these tests give positive reactions it becomes virtually certain that proteins are present, for the different color reactions are due to different groups in the protein molecule and no substance except a protein will respond to any considerable number of them

The first tests we consider are not color reactions, but precipitation reactions which are characteristic of proteins We have already mentioned that proteins are precipitated by saturated solutions of ammonium sulfate, zinc sulfate, and other salts These precipitates are usually soluble if the precipitate is filtered off and water or dilute salt solutions added Proteins may also be precipitated by the salts of heavy metals such as copper sulfate, lead acetate, and mercuric nitrate It is likely that in many cases these precipitates are the protein salts of the metal cations Proteins are also precipitated by alkaloid reagents such as picric acid, phosphotungstic acid, and tannic acid

The Biuret Reaction

If a protein solution is mixed with sodium hydroxide solution and a very weak solution of copper sulfate, a violet color, or in some cases a much more intense blue color than could be explained by the small amount of copper sulfate used, is obtained This test probably depends upon one or more of the following group of the protein molecule two (—CONHR) groups, or one (—CONHR) plus one of the following —$CSNH_2$, —$C(NH)NH_2$, —CH_2NH_2, —$CHRNH_2$, —$CHOHCH_2NH_2$, —$CHNH_2CH_2OH$, —$CHNH_2CHROH$

Millon's Reaction

Millon's reagent is prepared by dissolving mercury in nitric acid and represents a solution of mercuric nitrite and mercuric nitrate in a mixture of nitric and nitrous acid If a protein is heated with this reagent, a red color or precipitate is obtained, even with solid proteins The test is due to the presence in the protein molecule of the phenol group of tyrosine

The Hopkins-Cole (Glyoxylic Acid) Reaction

If a protein solution is mixed with glyoxylic acid and concentrated sulfuric acid is poured in to form a layer on the bottom, a violet ring is ob

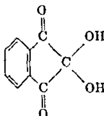
[illegible]

The Xanthoproteic Reaction

Xanthoproteic simply means "yellow protein" and refers to the fact that if a protein is heated with nitric acid a yellow color is obtained This is evidently due to nitration of the benzene ring in phenylalanine, tyrosine, or tryptophane Addition of ammonia after this heating causes the yellow color to become more intense and to shift towards the orange

The Ninhydrin (Triketohydrindene Hydrate) Reaction

When proteins are boiled with ninhydrin



a blue color is obtained This reaction is due to the α amino acid groups present in the protein molecule Naturally it is also obtained with the α amino acids themselves

REFERENCES

1 Anson, M L, and Edsall, J T *Advances in Protein Chemistry* Academic Press, Inc, New York, 1945
2 Clark, W M *Topics in Physical Chemistry* The Williams & Wilkins Co, Baltimore, 1948
3 Cohn, E J and Edsall, J T *Proteins, Amino Acids and Peptides* Reinhold Publishing Corp, 330 W 42nd St, New York, 1943
4 Cohn, E J, and Hughes, W L 1946 See Edsall, J T The plasma proteins and their fractionation Adv Protein Chem, 3, 457, 1947
5 Cohn E J, *et al* Chemical, clinical, and immunological studies on the products of human plasma fractionation I J Clin Investigation, 23: 417–432, 1944
6 Donnan, F G The theory of membrane equilibrium in the presence of a non diffusible electrolyte Zeitschr Elektrochem, 17: 572, 1911
7 Gibbs W On the equilibrium of heterogeneous substances Trans Conn Acad emy, 3 108–248, 343–524 1875–1878 See *The Collected Works*, p 55–371 Longmans, Green and Co New York 1928
7a Haurowitz F *Chemistry and Biology of Proteins* New York, N Y, Academic Press 1950
8 Hitchcock D I *Physical Chemistry* Charles C Thomas, Springfield Ill, 1934
9 Howe P E The function of the plasma proteins Physiol Rev, 5, 439–476, 1925

10 Kirk, P L The chemical determination of proteins Adv. in Prot Chem, 3 139–167, 1947.

11 Landsteiner, K, and van der Scheer, J On cross reactions of egg albumin sera J E M, 71: 445–454, 1940

12 Lloyd, D J, and Shore, A *Chemistry of the Proteins* P Blakiston Co, Phila 1938

13 Loeb, Jacques *Proteins and the Theory of Colloidal Behavior* McGraw Hill Book Co, New York, 1922

15 MacInnes, D A *The Principles of Electrochemistry* Reinhold Publishing Corp, New York, 1939

16 Northrop, J H *Crystalline Enzymes* Columbia Univ Press, New York, 1939

17 Popper, H, *et al* Chemical versus electrophoretic separation of proteins Am J Clin Path, 20: 530–538, 1950

17a Sharp, D G, *et al* The electrophoretic properties of serum proteins J Biol Chem, 144: 139–147, 1942

18 Sumner, J B and Somers, G F *Chemistry and Methods of Enzymes* Academic Press, Inc, New York, *1943*

19 Svedberg, T, and Pedersen, K O *The Ultracentrifuge* Oxford, Clarendon Press, 1940

19a Svensson, W Q Fractionation of serum with ammonium sulfate and water [illegible] 1941

20 [illegible] procedure for the [illegible] 730, 1948

21 [illegible] I Zein Jour

CHAPTER 2

Protein Structure

In a very real sense, life may be looked upon as a struggle on the part of the body to maintain the internal structure of the enormously complex protein molecules of which it is composed. The difficult nature of such a task may be judged from the fact that a distinctive property of most protein molecules is their extreme instability as compared with other chemical structures. Environmental factors as mild as the warmth of a human hand or the gentle bubbling of air would suffice, in many cases, to so alter the properties of a protein solution as to render it biologically useless.

That life should be built on such fragile, almost evanescent molecules is not at all surprising. It would seem, upon reflection, to be inevitable. Life implies change—quick adjustments to altered conditions. There must be something then in a living organism which can vary with the absorption of a few quanta of light, with trifling changes in air pressure, oxygen concentration, temperature, or any of the other hundreds of variables that beset us every moment of time. That something is the protein molecule.

It might be tempting for beginning students to equate complexity of structure with mere size of molecule. Thus, the β lactoglobulin of milk has an empirical formula which is thought to be $C_{1864}H_{3012}N_{468}S_{21}O_{576}$. Here we have a molecule in which we can count almost six thousand individual atoms of five different kinds. The molecular weight is over forty thousand, which means that the molecule is some twenty three hundred times as heavy as a water molecule and more than two hundred times as heavy as a molecule of the amino acid, tryptophane. And yet β lactoglobulin is a protein of comparatively simple structure. Certainly, its molecular weight is well below the average for proteins. Molecular weights in the hundreds of thousands are common and those in the millions are not unknown. There are protein molecules, in other words, which compare with β lactoglobulin as that protein compares with tryptophane.

Yet, knowing size, we know comparatively little. There are other types of molecules produced by living organisms which compare with proteins in molecular weight. There is cellulose, for instance, impressive in size, and yet used for nothing more in the living plant than to enclose the cell in a sturdy box. It is a huge molecule, yet so stable that we build houses out of

Size alone is therefore no guarantee that a molecule will possess the flexibility and instability needed to have within it the potentiality of life

PROTEINS AS AMINO-ACID CHAINS

The Nature of The Chain Unit

In considering protein structure, we might *for the moment focus our attention* upon a typical protein molecule as compared with a molecule of cellulose Both are alike in that they are repetitive structures That is, they are constructed of repetitions of comparatively simple units, somewhat in the manner that beads are strung together to form a necklace

Both proteins and cellulose can be so treated as to be broken down into smaller chains or into the ultimate mixture of the units themselves Cellulose, after strenuous hydrolysis with mineral acids, is broken down to molecules of glucose, a simple sugar with a molecular weight of 180 In order to build the cellulose molecule, as many as two thousand glucose molecules may be condensed (with loss of water) Under acid or alkali hydrolysis, a protein molecule is similarly degraded to its units, the amino acids discussed in Chapter 1, and here again thousands may be condensed with one another to build a protein But there is an important difference to be noted Whereas cellulose possesses a single kind of unit, glucose, proteins possess more than twenty different units, all amino acids to be sure, but differing among themselves otherwise

This difference is characteristic of proteins Other repetitive structures either natural or synthetic, are built up of not more than one or two different units (nucleic acids are a partial exception—see Chapter 7), and here would seem to be one of the answers to the problem of protein complexity *Whereas in the case of cellulose, for instance, individual molecules can vary* only in the number of glucose units in the chain, in the case of proteins, not only the number but the *type* and *arrangement* of the units becomes significant The difference implied here is somewhat analogous to that existing between an artist compelled to paint a scene in a single color and one who is allowed twenty colors

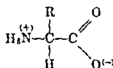
The first task in investigating protein structure is then not to count atoms but to investigate the manner in which amino acids are combined

The Peptide Linkage

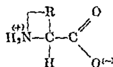
The problem of the manner in which amino acid combinations take place in the formation of proteins would be impossibly complicated if each amino acid could utilize its reactive potentialities to the full Each amino acid, for instance contains an amino group and a carboxyl group both quite active chemically In addition more than half of the amino acids possess other groups of active chemical nature

The situation is simplified, however, by the fact that it is easy to show that the various linkages between amino acids are of similar chemical nature That is, it is not very likely that tyrosine will combine with lysine via the carboxyl group of the first and the alpha amino group of the second at one time, then via the phenolic group of the first and the epsilon amino group of the second at another time If there were such variations, then some portions of the amino acid chain would be much more easily split off than other portions, depending upon the comparative strengths of the various types of bonds

This is not so In hydrolysis, it is found that under given conditions of acid (or alkali) strength, or of temperature, the various amino acid linkages are broken with fairly similar ease It is very reasonable then to suppose that whatever the inter amino-acid bonds are, they are equivalent and must



I Amino acid



II Imino acid

involve groups that are common to all the amino acids without exception These, of course, can be only the carboxyl and α amino groups It will only be necessary to consider the amino acid molecule, therefore, in the general ized form shown in formula I, or, in the exceptional case of proline and hydroxyproline, as in formula II

There are two ways in which amino acids can be combined so that only the indicated groups are involved and all linkages are equivalent One of these would be an electrostatic link (salt formation) in which the positive pole of each amino acid would attract the negative pole of its neighbor [illegible]

amino acids at once, as they ought to do if the above hypothesis were [illegible]

the elimination of one molecule of water per amino acid in the manner indicated in formula III

That this is the actual linkage between the amino acids in the protein molecule is as well established as anything can be in protein chemistry The linkage (—CO—NH—) is known as a *peptide bond* Groups of amino acids combined in this manner are called *dipeptides*, *tripeptides*, and so forth, according to the number of amino acids in the chain Where the chain is long and the number of amino acids composing it is indeterminate, the generic term *polypeptide* is applied The amino acids in the chain being minus the elements of water are termed *amino acid residues*

The lines of evidence pointing to the existence of the peptide linkage are many and conclusive Some of the evidence may be indicated

a Proteins in their natural state contain far fewer free amino groups or free carboxyl groups than would correspond to the number of amino acid residues they contain, indicating these groups to be involved in bond formation Such free amino groups as are detected approximate the number of lysine residues contained by the protein This is to be expected since lysine possesses a second amino group not concerned in the peptide linkage Similarly free carboxyl groups result from the presence of aspartic acid and glutamic acid in the molecule Furthermore when proteins are hydrolyzed amino groups and carboxyl groups are invariably liberated in equivalent quantities, exactly as would be expected if the hydrolysis consisted of the breaking of peptide bonds by the addition of the elements of water

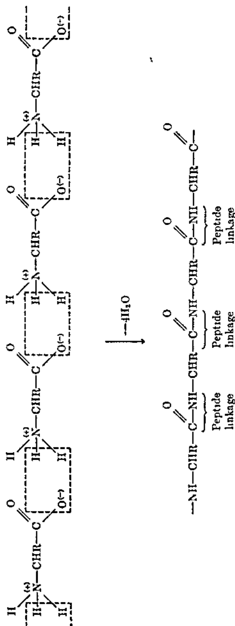
b Protein fragments more complicated than amino acids can be isolated after incomplete hydrolysis These frequently turn out to be identical chemically with known synthetic short chain peptides Thus, glycyl tyrosine has been isolated from the incompletely hydrolyzed fibrous protein of silk (formula IV)

c Enzymes which catalyze the hydrolysis of proteins in the digestive tract, act in similar fashion upon synthetic peptides In view of the specificity of enzyme action (see Chapter 5), this is excellent evidence in favor of the peptide linkage

d The absorption spectrum produced by the passage of infra red radiation through protein films has been found to show an appearance similar to the spectra of other substances known to contain the —CO—NH— linkage (16)

X-Ray Diffraction

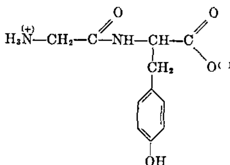
X rays have proven themselves powerful tools in the investigation of the structure of some of the simpler protein molecules Through their use it has been possible to estimate the distance between the various atoms of the polypeptide chain (2, 11)



III Polypeptide chain

It is not proposed here to go into the theory of x ray diffraction in detail It need only be said that when a parallel beam of x rays impinges upon any substance, the constituent atoms of that substance become centers for the scattering of radiation Where the atoms are randomly placed, as in amorphous material, scattering is likewise random, and if a photographic plate is placed behind the material, one will detect a dark central spot due to that portion of the x ray beam which pierced the substance undeflected Surrounding it will be a hazy, featureless circle of scattered radiation, fading as the distance from the central spot increases

However, where an orderly array of atoms is presented to the x ray beam as in the case of crystals, planes of atoms exist which reinforce each other's scattering properties, so that portions of the impinging beam are deflected as subsidiary beams In this case, the central spot on the photo



IV Glycyl tyrosine

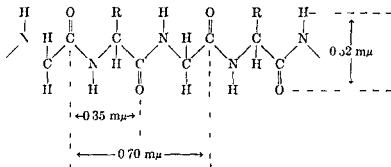
graphic plate would be surrounded not by a featureless haze, but by well defined spots located in beautifully symmetrical fashion From the position of these spots it is possible to determine the relationship of the planes of similar atoms existing within the crystal, in terms of actual distance

It is not necessary that material be crystalline in the ordinary sense for x ray diffraction to be of use It is only necessary that there be present periodically repeated chemical groups Thus such non crystalline substances as silk, hair, and cotton are suitable for x ray diffraction studies since they consist of protein or cellulose which are polymers made up of repeating units If silk fibroin is the protein studied, the data obtained are consistent with the view that the molecule is a puckered polypeptide chain of the dimensions shown in formula V The chain, as thus pictured, is a fully extended one It might seem as though there were room for further extension to allow the carbon and nitrogen peptide backbone to become completely linear Because of the arrangement in space of the valence bonds about the carbon and nitrogen atoms, such an extension is impossible The

question of the spatial configuration of the valence bonds of carbon will be discussed in detail later in the chapter

Silk fibroin, which is an inelastic protein, maintains its configuration under all ordinary conditions The protein of hair, keratin, which is elastic presents a somewhat more complicated case When wetted, it can be stretched to as much as three times its original length When fully extended in this fashion x ray diffraction patterns indicate a structure similar to that of silk fibroin In its normal contracted state the polypeptide backbone of keratin forms sinuous loops so that the molecule as a whole becomes shorter and thicker

Since 1947, artificial polypeptides have been synthesized with molecular weights nearly in the protein range These have been investigated by x ray



V Silk fibroin

diffraction and have been found to yield results similar to those of the natural fibrous proteins (6)

It should be emphasized here that most of the information obtained by x ray diffraction has been with reference to fibrous proteins These proteins are, structurally, much simpler than the non fibrous, or globular proteins This is a point that will be taken up in greater detail later on in the chapter

Quantitative Determinations of Protein Amino Acids

Having decided on the manner in which amino acids are combined in the protein molecule and having obtained some notions as to the size and shape of the polypeptide backbone in some of the less complex proteins, there remains the problem of determining exactly the numbers of the several amino acids in the molecule and, if possible, their arrangement as well A number of techniques have been evolved for the purpose of investigating this problem of amino acid content of proteins

Chemical methods. These are the oldest, and, currently the least used and least useful means of analyzing for amino acids It is not necessary to

outline the many tests that have been employed by various workers to determine the various components of the hydrolysis mixture of proteins These can be found by the interested student in a monograph such as that of Schmidt (25) Many of these methods depend upon the formation of derivatives which may be fractionally distilled (as the ethyl esters of the amino acids) or fractionally precipitated (as in the case of uramino or hydantoin derivatives) Individual amino acids may be precipitated from the mixture (as, for instance, arginine and histidine in the form of silver salts) or determined by a more or less specific colorimetric reaction while still forming part of the mixture as tyrosine is, through use of a phosphomolybdate solution Most of the tests used are tedious in nature and suffer from a lack in either quantitativeness, specificity, or both

Spectrophotometric methods Spectrophotometric analysis depends on the ability of chemical substances to absorb selectively specific wave lengths of light The reason why certain wave lengths and not others are absorbed by a given compound lies in the field of quantum mechanics and is beyond the scope of this text The fact of this selective absorption is however, familiar to all of us from youth Colored glass for instance, is colored because it will transmit some wave lengths of light and absorb others Inorganic ions such as cupric ion or chromate ion are colored for the same reason, as are the many organic coloring matters both natural pigments and synthetic dyes

Those substances which to us appear colorless also absorb light but at wave lengths in the ultra violet or infra red where the effects must be detected by photocells or by the photographic plate The spectrophotometer is an instrument designed to measure the amount of this light absorption Essentially, the principle on which it is based may be described as follows A beam of visible or ultra violet monochromatic light falls upon a photocell after passing through a cell of standard dimensions containing the solvent

... to be investigated will later be dissolved The elec

he light passes

nd to be inves

e difference in

current intensities may be read directly as per cent light transmitted or *percentage transmittance*

From percentage transmittance, one can obtain *absorbency*, formerly called optical density or extinction as follows

... t alone

absorbency =

Since the percentage transmittance of the solvent is usually arbitrarily set

at 100, the equation becomes

$$\text{absorbency} = \log \frac{100}{\text{percentage transmittance}}$$

$$= 2 - \log (\text{percentage transmittance})$$

Many instruments are so devised as to allow absorbency to be read directly

The advantage of absorbency over the apparently simpler and more straightforward percentage transmittance lies in the fact that in the case of most substances *absorbency varies directly with concentration* all other factors being equal This is a statement of *Beer's Law* which is so fundamental in analyses by optical methods and so useful that there is a tendency to forget that it does not always hold true For Beer's Law to be valid there must be no change in the chemical nature of the substance as the concentration is changed, no association nor dissociation of molecules no significant changes in per cent ionization nor any reaction with the solvent In photometric analyses it is advisable therefore to plot standard calibration curves first This is done by measuring the absorbencies of *known* concentrations of the substance being studied at the wave length being used Usually it is found that over some range of concentrations the relationship is virtually linear (i e, Beer's Law holds) Outside that range, photometric measurements can be valid only when comparative readings are made with standard solutions closely approximating the concentrations of the unknowns

In photometric analyses another factor of great importance is the wave length of light used in measuring absorbencies It seems obvious upon reflection that the most useful wave length is the one at which the absorbency varies most with concentrations since here the method would be most sensitive This point is usually (but not invariably) at a wave length where absorption is greater than it is at lower and higher wave lengths, that is, at absorption peaks

Both the location of the absorption peak and its intensity per unit concentration are characteristic of the electronic make up of the substance being investigated While the subject, if treated rigorously, is most complex, certain generalizations can be made which are both simple and useful for beginners Among organic compounds it is a general rule that unsaturated compounds show absorption peaks at longer wave lengths than do saturated compounds This is particularly true if the unsaturated bonds are conjugated (i e if single and double bonds alternate in the carbon chain) Thus, in the case of benzene there is an absorption peak at about 270 mμ, whereas in the case of hexane no absorption peaks of any significance occur until wave lengths well below 200 mμ are reached

Of the amino acids contained in proteins three contain the benzene

ring These are phenylalanine, tyrosine, and tryptophane (To these, the specialized amino acid, thyroxine, may be added, but except in proteins of the thyroid gland, its possible presence can be neglected) These amino acids show absorption peaks in the region 260–290 mμ whereas all other amino acids are almost perfectly transparent there

It is thus possible to determine the aromatic amino acid content of proteins without the necessity of any separatory procedures on the hydrolysis mixture (15) One can indeed determine it in the intact protein In fact the tyrosine tryptophane phenylalanine absorption band has been used for the determination of protein content of solutions (10)

While spectrophotometry has the virtue of simplicity and elegance, there are serious drawbacks as far as its use in amino acid analysis is concerned In the first place, the method is restricted to the three amino acids mentioned Secondly, it can not very well distinguish among the three Although the absorption peak is slightly different in each case, it is to be remembered that a peak is never a sharp upsurge of absorbency at a given wave length but is rather merely the most pronounced point of a fairly broad region of absorption In the case of tyrosine, tryptophane and phenylalanine, the three regions of absorption overlap badly, so that it is difficult to determine how much of the observed absorption is contributed by each of the three without the use of subsidiary methods of analysis Finally, there exist common cellular constituents (chiefly the purine and pyrimidine bases of the nucleic acids—see Chapter 7) in close association with proteins, which absorb in the same region of the ultra violet spectrum with an intensity some ten times as great as that of the aromatic amino acids The possibility of a contamination by even small quantities of such substances is therefore very serious.

Isotopic methods Recent years have brought an extremely powerful tool to the aid of the biochemist in the form of isotopes (see Appendix II) Isotopes of a given element are atomic species which differ among themselves only in the number of neutrons in their nuclei The number of protons in the nucleus and consequently the number of planetary electrons in the neutral atom remains the same The result is that the chemical properties of the various isotopes of a given element (which depend upon the number of electrons in the neutral atom) are virtually identical On the other hand, isotopes of a given element can be distinguished one from the other by various physical methods Particularly, where one isotope is radioactive it can be detected by radiation counting devices which have an almost fantastic sensitivity In this manner it can be distinguished from its non radioactive isotopic brother, with which it still shares in common all chemical properties Furthermore, the radioactive properties

of a given isotope, let us say C^{14}, are not in the least affected by the incorporation thereof into a molecule such as an amino acid

If now, a known quantity of an amino acid such as arginine containing C^{14} as part of its structure is added to a protein hydrolysate and the mixture stirred to homogeneity, the ratio of stable and radioactive arginine will remain the same at all stages of chemical isolation Once separated in pure form (not necessarily quantitatively) the proportion of the mixture which is radioactive can be determined Since the ratio is just what it was at the start and since the total quantity of radioactive arginine added is known the quantity of arginine in the original hydrolysate is easily calculated

Although this *isotopic dilution method* (26) is quite accurate and is applicable to any amino acid, it has certain practical drawbacks There is always the necessity of isolating the given amino acid in great purity, and this is sometimes difficult Furthermore radioactive amino acids and equipment used in dealing with radioactivity are quite expensive and safety precautions that are not required in other analytical techniques must be taken in any procedures involving the use of radioactive substances

Nevertheless, despite expense and the special precautionary measures involved isotopic procedures are becoming more prominent yearly in biochemical research, particularly in the study of metabolism

Enzymatic methods. The use of enzymes in analyzing for individual amino acids in a hydrolysate (1) is not very different in principle from the use of a specific chemical precipitant Enzymes are proteins which catalyze certain reactions in a very specific way (see Chapter 5), and the advantage of their use lies in this specificity

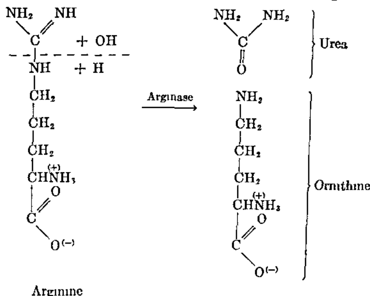
Thus an enzyme known as arginase will, under the proper conditions of pH, split urea from arginine in the manner shown in formula VI Arginase will not affect any other amino acid in the slightest so that a measure of the rate of urea formation is an accurate representation of the amount of arginine originally present

Unfortunately, so convenient an enzymatic reaction can not be found for every amino acid In addition, enzymatic methods of analyses have their own *difficulties of technique as will be explained fully in Chapter 5*

Microbiological methods. The use of enzymes not as separate entities but as parts of living micro-organisms is gaining in popularity The basic principle underlying their use in amino acid analysis is the fact that strains or varieties of certain micro organisms can often be cultivated which require for normal growth the presence of a specific amino acid in their culture medium (27)

A typical micro-organism so used is *Neurospora*, a common mold In its

natural wild form it can grow normally upon a medium containing certain necessary minerals water, and a small quantity of the organic food factor, biotin From these plus carbon dioxide *and oxygen, it can manufacture* in quantities suitable for its needs all the amino acids Exposure of *Neurospora* to ultra violet or x radiation, however, may cause mutations to arise through disturbances within the genetic factors of the cell (see Chapter 9) These mutations demonstrate themselves often in a disability to manufacture *some compound vitally needed for metabolism* The compound required is usually a vitamin or an amino acid As far as a given mutant



VI

strain is concerned, the compound it can not synthesize must be supplied *in its culture medium*, and over a certain range the rate of growth is proportional to the concentration of the needed factor in its food supply

If, then, one had a supply of a strain of *Neurospora* which could not manufacture lysine from inorganic constituents (a so called lysine less" strain), one need only grow it upon a medium containing in part the protein hydrolysate to be analyzed, and then measure its rate of growth to determine the concentration of lysine

The difficulties here lie largely in the techniques required to grow the micro organism and in forming, *isolating, and identifying the useful mutant* strains Moreover, strains useful in analyzing each of the amino acids can not be obtained with equal ease In the case of some amino acids, i e, cystine, the appropriate mutant strain is rarely or never met It should

be stressed that the strains arise not through any purposeful action on the part of the experimenter but through the random and unpredictable actions of high-energy radiation

Chromatographic methods. Chromatography, in its various phases is now nearly half a century old Originally, it was a technique by which closely related compounds were separated through their differing tendencies to be adsorbed on a material such as powdered aluminum oxide Earliest experiments were made on such plant pigments as the chlorophylls and the carotenoids When these (in solution) were passed through a column packed with aluminum oxide, the individual compounds of the mixture were adsorbed and held by the powder Those compounds which were strongly adsorbed clustered at the very top of the column, while those with somewhat less tendency for adsorption percolated through the column a longer distance before surface forces held them Further addition of pure solvent to the column tended to wash the mixture downward Again, the component which was less strongly held by the alumina was more easily loosened and was washed down further than the component which was more strongly held Eventually the bands representing the various components of the colored mixture were entirely separate and visible to the eye as varicolored regions in the column Hence the original name chromatographic adsorption' The components of the mixture, through adsorption, are ' written in color" This technique is not restricted to visibly colored compounds Actually, any mixture for which the appropriate solvents and adsorbents can be found can be chromatographed Where the components can not be readily distinguished by eye as in the case of amino acids it is only necessary that more indirect chemical methods be used Many natural and synthetic substances besides aluminum oxide are used in chromatography Starch in particular has been used recently with considerable success as a chromatographic medium for amino acids (20) Its use does not vary in principle from that of aluminum oxide

A great many synthetic polymers have since been devised for the specific purpose of facilitating certain separations These polymers depend for their action, not upon the surface phenomenon of adsorption, but upon actual chemical reactions with the mixture passing through the column Thus a given polymer may, at a pH of 7 for instance, contain many carboxylate ions These will attract, by electrovalent forces, compounds sufficiently basic to be positively charged at pH 7 Furthermore the place in the column at which the ester of a given component will tend to cluster will depend on how strongly basic it is Of two components, one of which is slightly more basic than the other, the more basic one will be found higher in the column The addition of stronger base by replacing the compounds being investigated, washes or ' elutes" them down through

the column, again replacing the least basic most easily If the liquid emerging from the column is collected in small fractions, it is found that the various fractions will contain portions of single components of the mixture The polymer can then be regenerated for further use by passing buffer at pH 7 through it Other polymers can be designed to possess basic groups which will separate acidic components of a mixture, or indeed to possess groups specifically intended to take advantage of almost any peculiar chemical properties of the substances being studied These polymers are known as *ion exchange resins* and such is their efficiency that they have been used to de salt water, the water so obtained being as salt free as ordinary distilled water

The type of chromatography, however, which has been applied most fruitfully to the analysis of complex amino acid mixtures is known as *paper partition chromatography* (29) This has become an extremely popular technique for amino acid analysis and for other problems of similar nature

In paper partition chromatography advantage is taken of the different partition coefficients of two closely related substances The partition coefficient may be defined as the ratio of the solubilities of a substance in two different mutually insoluble solvents If for instance two amino acids in aqueous solution are shaken with butyl alcohol in a separatory funnel and the two phases allowed to separate, both amino acids will be found not only in the aqueous layer but in the butyl alcohol layer as well The amount of each in each layer depends upon the solubilities of each amino acid in the two solvents Let us suppose that amino acid A is more soluble in water than is amino acid B, and conversely less soluble in butyl alcohol Another way of saying this is that the water/butyl alcohol partition coefficient of amino acid A is greater than that of amino acid B The result of all this is that when an aqueous mixture of the two has been shaken with butyl alcohol, the proportion of amino acid B in the butyl alcohol layer will be higher than it was in the original mixture, while in the aqueous layer the proportion of amino acid B will be lower

One can well imagine that if the aqueous layer is extracted a second time with butyl alcohol, and a third and a fourth *ad infinitum*, eventually only amino acid A will be left in the water, and that if the butyl alcohol layer is extracted and re extracted with water, only amino acid B will be left there To be sure, only vanishingly small quantities of either will be left after such treatment, but nevertheless this principle has been put to use in what is known as the countercurrent technique (8) Here a series of separatory funnels or their equivalents are used in many successive extractions, so that each component of a mixture is spread throughout the funnels at varying rates and may thus be separated This technique, however, is

somewhat cumbersome and has not been applied to any great extent to amino acid separation

In amino acid separations (7) a strip of ordinary filter paper takes the place of the separatory funnels A drop of the mixture to be separated is placed near one end of the filter paper strip and allowed to dry That end is then dipped into butyl alcohol (which has been water saturated) and the liquid allowed to travel through the filter paper by capillary action past the spot where the dried mixture has been placed The components of the mixture are now under the influence of two tendencies On the one hand they have the tendency to stay where they are, remaining dissolved ' in the water film which is adsorbed on the surface of the filter paper (This water film is present under all ordinary circumstances, and its removal would require prolonged desiccation of the filter paper) On the other hand, there is the tendency to be dragged along by the advancing butyl alcohol The extent to which a given amino acid follows each of these tendencies depends upon its comparative solubilities in water and in butyl alcohol A highly water soluble amino acid such as lysine would be bound comparatively strongly to the water film and would move but slightly with the butyl alcohol front A comparatively water insoluble amino acid such as phenylalanine on the contrary, will be bound weakly to the water film and will move comparatively rapidly with the butyl alcohol The tendency of the amino acids thus to separate is accentuated with time and eventually the amino acids which were originally all superimposed upon the original drop of mixture will be strung out in various positions along the filter paper

It is of course not impossible that two amino acids will travel at so nearly the same rate that even after hours of separation they will remain at least partially superimposed A second separation is therefore usually performed After allowing the filter paper to dry, it is rotated through ninety degrees dipped into a second solvent (say water-saturated phenol) in such a way that the line of amino acids now lies horizontally an inch or so above the surface of the phenol As the phenol rises past each spot there is again a separation, and if anywhere along the line two or three amino acids are superimposed it is extremely unlikely that their phenol water partition coefficients will also be equal as their butyl alcohol/water partition coefficients were As a matter of fact, after such a two-dimensional separation, no two amino acids will be found occupying the same spot on the filter paper A complete separation of all naturally occurring amino acids can be performed thus

The position of the spots is of course invisible to the naked eye The spots can be visualized by allowing the filter paper to dry and then spraying it with ninhydrin solution and warming Wherever an amino acid is located

the column again replacing the least basic most easily If the liquid emerging from the column is collected in small fractions it is found that the various fractions will contain portions of single components of the mixture The polymer can then be regenerated for further use by passing buffer at pH 7 through it Other polymers can be designed to possess basic groups which will separate acidic components of a mixture or indeed to possess groups specifically intended to take advantage of almost any peculiar chemical properties of the substances being studied These polymers are known as *ion exchange resins* and such is their efficiency that they have been used to de salt water the water so obtained being as salt free as ordinary distilled water

The type of chromatography however which has been applied most fruitfully to the analysis of complex amino acid mixtures is known as *paper partition chromatography* (29) This has become an extremely popular technique for amino acid analysis and for other problems of similar nature

In paper partition chromatography advantage is taken of the different partition coefficients of two closely related substances The partition coefficient may be defined as the ratio of the solubilities of a substance in two different mutually insoluble solvents If for instance two amino acids in aqueous solution are shaken with butyl alcohol in a separatory funnel and the two phases allowed to separate both amino acids will be found not only in the aqueous layer but in the butyl alcohol layer as well The amount of each in each layer depends upon the solubilities of each amino acid in the two solvents Let us suppose that amino acid A is more soluble in water than is amino acid B and conversely less soluble in butyl alcohol Another way of saying this is that the water/butyl alcohol partition coefficient of amino acid A is greater than that of amino acid B The result of all this is that when an aqueous mixture of the two has been shaken with butyl alcohol the proportion of amino acid B in the butyl alcohol layer will be higher than it was in the original mixture while in the aqueous layer the proportion of amino acid B will be lower

One can well imagine that if the aqueous layer is extracted a second time with butyl alcohol and a third and a fourth *ad infinitum* eventually only
layer
left th
left after such treatment but nevertheless this principle has been put to use in what is known as the countercurrent technique (8) Here a series of separatory funnels or their equivalents are used in many successive extractions so that each component of a mixture is spread throughout the funnels at varying rates and may thus be separated This technique however, is

somewhat cumbersome and has not been applied to any great extent to amino acid separation

In amino acid separations (7), a strip of ordinary filter paper takes the place of the separatory funnels A drop of the mixture to be separated is placed near one end of the filter paper strip and allowed to dry That end is then dipped into butyl alcohol (which has been water saturated) and the liquid allowed to travel through the filter paper by capillary action past the spot where the dried mixture has been placed The components of the mixture are now under the influence of two tendencies On the one hand they have the tendency to stay where they are, remaining dissolved" in the water film which is adsorbed on the surface of the filter paper (This water film is present under all ordinary circumstances, and its removal would require prolonged desiccation of the filter paper) On the other hand, there is the tendency to be dragged along by the advancing butyl alcohol The extent to which a given amino acid follows each of these tendencies depends upon its comparative solubilities in water and in butyl alcohol A highly water soluble amino acid such as lysine would be bound comparatively strongly to the water film and would move but slightly with the butyl alcohol front A comparatively water insoluble amino acid such as phenylalanine, on the contrary, will be bound weakly to the water film and will move comparatively rapidly with the butyl alcohol The tendency of the amino acids thus to separate is accentuated with time and eventually the amino acids which were originally all superimposed upon the original drop of mixture will be strung out in various positions along the filter paper

It is of course, not impossible that two amino acids will travel at so nearly the same rate that even after hours of separation they will remain at least partially superimposed A second separation is therefore usually performed After allowing the filter paper to dry, it is rotated through ninety degrees, dipped into a second solvent (say, water saturated phenol) in such a way that the line of amino acids now lies horizontally an inch or so above the surface of the phenol As the phenol rises past each spot there is again a separation, and if anywhere along the line, two or three amino acids are superimposed it is extremely unlikely that their phenol/water partition coefficients will also be equal as their butyl alcohol/water partition coefficients were As a matter of fact, after such a two-dimensional separation, no two amino acids will be found occupying the same spot on the filter paper A complete separation of all naturally occurring amino acids can be performed thus

The position of the spots is of course invisible to the naked eye The spots can be visualized by allowing the filter paper to dry and then spraying it with ninhydrin solution and warming Wherever an amino acid is located

a visible spot will appear By conducting experiments where each amino acid is treated singly in this manner a map of spots can be developed so that through the R_f values (the ratio of the rate of movement of a given spot to that of the solvent boundary) one can, by noting the position of the spots determine the identities of the amino acids present in the mixture

Paper chromatography is simple sensitive, and inexpensive For most accurate results certain precautions must be taken Thus of the two solvents used one is usually water although this is not absolutely necessary since by first washing the filter paper intensively with another solvent a standing solvent other than water can be used The other must be a solvent which is not entirely miscible in water, and which is sufficiently similar chemically so that the compounds being investigated will be appreciably soluble in both Butyl alcohol and water are an ideal such pair The separation must be conducted *in a closed container where the atmosphere* can be saturated with both butyl alcohol and water in order that evaporation effects not affect the procedure For similar reasons the butyl alcohol used must be water saturated in order that it may not dissolve the adsorbed film of water on the filter paper and thus destroy the partition effect upon which the separation is based For best results separations should be conducted at constant temperature The filter paper itself plays a minor role and can be viewed merely as a support for the two liquid phases Nevertheless it too must be chosen with care It must be quite pure and quite homogeneous to prevent the production of annoying artifacts

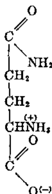
The great difficulty in paper chromatography lies in interpreting the results quantitatively A roughly quantitative notion as to amino acid concentrations can be obtained by measuring the area of each spot and the intensity of color The spots may also be compared with those resulting from the treatment of known quantities of the amino acid concerned It should be mentioned that through paper chromatography mixtures containing components present in quantities of only one to five micrograms (or even less in some cases) can be easily separated and the components accurately identified

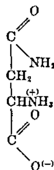
Amino Acid Contents of Proteins

All the techniques listed have been used in quantitative determinations of the amino acids in proteins Brand and his group (5) reported the amino acid composition of the protein β lactoglobulin mentioned at the beginning of this chapter They also proposed a shorthand notation for the various amino acids involving usually the use of the first three letters of the common chemical name The results are shown in table 3a see p 88

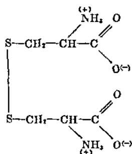
Two points should be mentioned in connection with the table First is

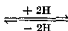
the presence of *glutamine* (formula VIIa) which is a derivative of glutamic acid This is the first indication that the side chains of the amino acids play a role in protein structure The role of the side-chain will be discussed in detail (Actually, amide nitrogen is here arbitrarily assigned to glutamic acid There is every reason to believe that the amide of aspartic acid, *asparagine* (formula VIIb), also occurs in proteins)

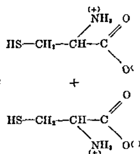




VIIa Glutamine VIIb Asparagine







VIIIa Cystine VIIIb 2 Cysteine

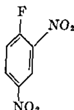
Secondly, there is the device of counting the number of *half* cystine molecules in the protein This is done because of the fact that cystine has in its molecule two amino groups and two carboxyl groups, thus giving the appearance of a double amino acid Furthermore, cystine (formula VIIIa) is easily converted to two molecules of cysteine (formula VIIIb) This reaction is an important one in protein chemistry and it is convenient to avoid changing the number of residues present Thus, two cysteine mole cules can be considered as being oxidized to two half-cystine molecules

Similarly such analyses of amino acid content have been made for other

proteins, for instance, for lysozyme, a protein found in tears which has a lytic (dissolving) effect upon bacteria The results of Fromageot's analysis of this protein (12) are also included in table 2

Unfortunately, such catalogues of amino acids, while representing an enormous advance are but a small part of the story There is still the question of arrangement of the amino acids in the peptide chain Methods for determining this experimentally are still inadequate and not surprisingly so in view of the tremendous complexity of the problem Beginnings have been made however

Sanger has introduced the use of 2,4-dinitrofluorobenzene (formula IX) in this connection (24) This reagent will form colored compounds with *free* amino groups and will thus add to any lysine residues present in the protein and to those amino acids at the end of a chain which possess a free amino group (A moment of reflection will be enough to see that at



IX 2,4 Dinitrofluorobenzene
(Sanger s reagent)

one end of a peptide chain is an amino acid with a free amino group, and at the other, one with a free carboxyl group) If the protein treated with Sanger s reagent is now hydrolyzed, the presence of the yellow dinitrophenylated amino acids can be detected and identified by paper chromatography If the presence of lysine is allowed for, one of the end amino acids on the chain can be determined In this manner, it has been found for instance that insulin (see Chapter 12) consists of a combination of four polypeptide chains, two of which end in glycine and two in phenylalanine

Another such end residue determination with perhaps greater potentialities has been introduced by Edman (9) He found that by reacting a peptide with phenylthiourea, a condensation compound is formed which can be split off from the rest of the chain by a mild hydrolysis which would not affect ordinary peptide linkages The fragments can be separated identified chromatographically, and the process repeated on what remains of the chain Presumably, amino acids could be identified one by one as they were unraveled from the chain given enough patience As yet, however, the method has been applied only to synthetic di and tripeptides

Another device for determining the arrangement of the amino acids

along the polypeptide chain is to hydrolyze a protein incompletely and to then identify the di and tripeptides in the hydrolysis mixture Assuming that the order of amino acids in the resulting peptides is the same as in the original protein the pieces can eventually be put together like a jigsaw puzzle Unfortunately it is a very complicated jigsaw puzzle indeed and the method has so far yielded only fragmentary results The most fruitful results have been obtained in the case of insulin (24a) Here it was found that the two peptide chains ending with glycine contain 21 amino acid residues including four of the six cystine residues present in the molecule These chains contain no lysine arginine histidine tyrosine phenylalanine or proline The remaining two peptide chains ending in phenylalanine contain 30 amino acid residues including the remaining two cystine residues A combination of careful partial hydrolyses and chromatographic separations resulted in the determination of the exact order of occurrence of all 30 residues in the phenylalanine chains (formula X)

Even this admirable work represents an incomplete analysis So far the jigsaw has been completely put together only in the case of one of the simplest known naturally occurring polypeptides gramicidin S Gramicidin S

Phe val asp NH_2 glu NH_2 his leu CySSCy gly ser his leu val glu ala
ala lys pro thr tyr phe phe gly arg glu gly CySSCy val leu tyr leu

X Phenylalanine-ending peptide chain of insulin

is an antibiotic (a naturally produced substance with bactericidal or bacteriostatic properties) which was first reported by Russian workers—the S standing for Soviet On investigation (28) it turned out to be a polypeptide containing five kinds of amino acid residues Sanger s reagent yields no nitrophenylated residues after hydrolysis leading to the conclusion that gramacidin S has no end to its chain i e is a circular cyclopeptide From a study of the peptides obtained in incomplete hydrolysis the total structure is now considered to be using Brand s symbols

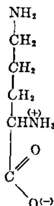
Leu Phe Pro Val Orn Leu Phe Pro Val Orn

The symbol Orn represents ornithine a diamino acid with one carbon less than lysine (formula XI) It is not found as a structural unit in human proteins but is considered to be an intermediary in the metabolism of arginine (see Chapter 14)

The phenylalanine (Phe) of gramicidin S is not quite the phenylalanine met with ordinarily in proteins It differs in the configuration of its atoms in space It is an optical isomer of ordinary phenylalanine and it would be well to pause now to explain what is meant by optical isomerism and to describe the role it plays in protein structure

Optical Isomerism

In considering the ordinary chemical formula as written on the black board or on a sheet of paper a great and unavoidable flaw enters into the symbolism since an attempt is being made to represent an object which exists in three dimensions upon a flat surface which can only show two In order to understand optical isomerism it is absolutely vital that the third dimension be taken into account Since it is not always easy to visualize three dimensions from two-dimensional representations, however



XI Ornithine



XII Tetrahedral carbon atom

painstakingly drawn and described, it would be advisable for students to follow the discussion below with ball and rod atom models

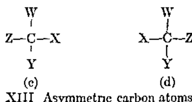
In space, the four valence bonds of carbon are *not* located in a single plane and directed toward the four corners of a square as usually represented Rather, the valences are located in two planes (a pair in each) which are mutually perpendicular As nearly as one can represent the three-dimensional situation on paper, the carbon atom may be depicted as in formula XII

In version "a" the solid bonds are to be visualized as sticking out in front of the paper, and making an angle of nearly 36° with the plane of the paper The dotted bonds, on the other hand, are sticking into and behind the paper again making a 36° angle with it The angle between *any two bonds* is a little over 109° In version ' b the two light solid bonds are in the plane of the paper The bold face solid bond sticks out from the paper

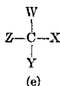
at an angle of about 55° and the dotted bond sticks into and behind the paper at the same angle (Actually if the two light solid bonds were in the plane of the paper, the other two bonds would be superimposed since they would lie in a plane perpendicular to that of the paper Their position in version "b" has been slightly distorted so that both might be shown) A little reflection, or better still, atom models, will prove to the student that the two versions, "a" and "b", are identical

This spatial configuration of carbon leads to important results when each of the four valence bonds of a carbon atom is attached to a group of a different nature Let us consider carbon atoms to which four such different groups, W, X, Y, and Z, are attached (formula XIII)

The question now arises whether any significant difference arises from the manner of attaching the four groups to the carbon atom In "c" and



XIII Asymmetric carbon atoms



XIV (d) turned through 180°

"d" for instance, we have added them first clockwise, then counterclockwise Does that matter? At first sight to the student trained in the planar representation of the carbon atom this would seem a frivolous question It would appear obvious to him that if one were to lift "d" up and out of the paper, turn it around so that the back is in front and put it back, he would have "c" and that therefore the two compounds are the same

Actually, this is not so where three dimensional configurations of carbon are concerned If we perform the process just described of lifting "d" out of the paper, reversing, and returning it, we end up with "e" (formula XIV) The placing of the attached groups in "e" would seem superficially to be the same as that in "c", but observe that in "e" X and Z stick *into* the paper, whereas in "c" they stick *out* of the paper Similarly, W and Y stick out of the paper in "e" and into it in "c"

On the basis of such considerations, the following statements can be made concerning the carbon atom

1 Where, of the four groups attached to a carbon atom not more than

three are different, then no matter how these four groups are arranged about the atom the molecules remain identical since movement in space can make any possible arrangement superimpose upon any other possible arrangement Such compounds are optically inactive

2 Where, of the four groups attached to a carbon atom *all four are different*, then there are two arrangements which can not be superimposed one upon another Furthermore, there are only two such non-superimposable arrangements, since all other conceivable arrangements can be reduced by motion in space to one of these two Such compounds are optically active, and the two isomers thereof are termed *enantiomorphs*

Again, it should be stressed that these statements are easily checked by the use of models

The further question now arises whether such pairs of optically active compounds as "c" and "d" possess different properties The answer to that is that chemically, except with respect to other optically active compounds such as enzymes, they do not, but physically they do Optically active compounds are asymmetric in that they possess no plane of symmetry, i e , no plane can be passed through the compound such that the portions on either side of the plane are mirror images of one another Such asymmetric compounds in solution rotate the plane of a beam of plane-polarized light passing through it It is for this reason that such compounds are termed optically active This phenomenon has given rise to an analytical technique known as polarimetry, which merits some discussion at this point

Light may be looked upon as behaving as though it consisted of trains of transverse waves (the exact nature of which together with the controversy as to whether light is wave like, particle like, or both in nature, may well be left to the realm of theoretical physics) Under ordinary circumstances, each photon of a beam of light restricts its undulations to a single plane, but since each photon "chooses" its plane of undulation at random, the end result is that waves occupy indiscriminately all planes passing through the line representing the direction of the beam Such a beam of light is termed unpolarized

When the waves of light are restricted to one plane, it is plane polarized There are many ways of achieving light polarization, and one which is perhaps used most frequently is to pass the light through a "Nicol prism" A Nicol prism is fashioned of a mineral known as Iceland spar, which is capable of refracting light in a double beam, both components of which are plane polarized in mutually perpendicular planes The prism is so designed that only one beam emerges therefrom, the other being totally reflected If a second Nicol prism is now placed so that the light issuing from the first must pass through it, then as long as the second prism is

aligned so that the molecular structure is parallel with that of the first all the light passes through As the second prism is rotated, less and less light can pass until, when the molecular structures of the two Nicol prisms are oriented perpendicularly, no light at all can pass through the second prism

By looking through the second prism, one can therefore determine the position of the plane of polarization by noting the position of the prism at the point where the light as seen through it is brightest (when it is parallel to the plane) or dimmest (when it is perpendicular to the plane)

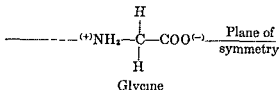
When a tube of distilled water is placed between the two Nicol prisms, no effect is noticed on the plane of light polarization If, however, a tube containing a solution of an optically active compound is placed between the two prisms, it is found that the second prism must now be rotated through a *different* angle to achieve either the position of maximum or of minimum light In other words, the optically active compound has rotated the plane of light polarization It has been found that when one of a pair of enantiomorphs rotates the plane a certain number of degrees clockwise or to the right, the sister compound rotates the plane the same number of degrees precisely, but in *counter*clockwise fashion or to the left That is the difference between the compounds "a" and "b" above

To the student meeting this concept for the first time it may seem that optically active pairs differ in a property whose nature is rather rarefied and theoretical It should be remembered, however, that these somewhat subtle optical differences are methods by which we can recognize very real and fundamental variations in spatial arrangements of atoms To living organisms these variations are vital Naturally occurring optically active compounds occur invariably as one of the other of the isomers—never as a mixture of the two The body differentiates between them and always prefers one isomer to the other

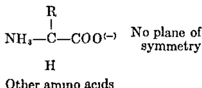
Spatial configuration of amino acids. Included in these naturally occurring, optically active compounds are all the amino acids but glycine If we look at the amino acids as methane derivatives, their optically active nature becomes apparent Of the four substituents on the central carbon atom of glycine only three are different There is a plane of symmetry and the molecule is optically inactive (formula XV) In the case of all other amino acids four different substituents are attached to the central carbon atom which thus fulfills the criterion of asymmetry and is optically active And of each pair of optically active amino acids the body uses but one

Which one? This is a question almost impossible to answer in an absolute way, that is, by actually indicating the position of each of the four substituents in space It can be answered relatively, however, by indicating how the various amino acids compare to one another In order to explain

this let us go back to the first investigations on optically active substances which were conducted on tartaric acid by Pasteur He discovered that one isomer of tartaric acid rotated the plane of polarized light to the right (clockwise) and one to the left (counterclockwise) Without going into the details of formulas, which would at this point add nothing to the principles expounded above, it will suffice to say that he therefore called the two isomers, "dextro tartaric acid" and "levo tartaric acid" These are usually written as D tartaric acid and L tartaric acid Similarly, other optically active substances were termed dextro or levo in accordance with the direction in which they rotated polarized light (Two common sugars are sometimes called dextrose and levulose for their possession of those very properties)



Glycine



Other amino acids

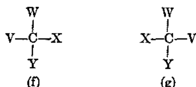
XV

However, it was soon discovered that the direction in which light was rotated was not necessarily dependent upon the spatial arrangement of atoms For instance let us suppose that of the two model enantiomorphs presented on page 67, "c" is dextrorotatory and "d" levorotatory If in each case, the group Z is replaced by another substituent V in a manner which is known not to affect the comparative position in space of the other three substituents (and this is by no means an easy thing to be sure of), compounds "f" and "g" are obtained (formula XVI)

Spatially, "f" is analogous to "c" and "g" to "d" It is therefore natural to assume that "f" like "c" is dextrorotatory, and "g" like "d" is levorotatory *This is not necessarily the case* One can not predict the direction of light rotation from the spatial configuration

It has been necessary for chemists to decide whether it was better to classify organic compounds on the basis of light rotation without regard to spatial configuration, or vice versa The second alternative was adopted since it was obvious that spatial configuration was the more fundamental

property as far as structural problems were concerned Glyceraldehyde was taken as the starting point (formula XVII) It is an optically active compound which may be viewed as a simple sugar (see Chapter 3) Since most of the research on optical isomerism has been done on the sugars it is a natural reference point The levorotatory isomer was termed *levo glyceraldehyde* (formula XVIIa) and the dextrorotatory isomer was termed dextro glyceraldehyde (formula XVIIb) No decision as to which isomer had which spatial configuration was made or was necessary Thereafter all compounds with asymmetric carbons that have the same spatial configuration as levo glyceraldehyde (as decided by the various tools at the disposal of the synthetic organic chemist) are termed levo compounds *regardless of the direction in which they rotate plane polarized light* The equivalent state-



XVI Spatial analogs of (c) and (d)



XVIIa L Glyceraldehyde XVIIb D Glyceraldehyde

ment may be made for compounds spatially related to dextro glyceraldehyde

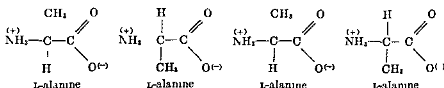
It is now common to distinguish between two dextro compounds that rotate light in opposite directions by means of an added plus or minus sign Thus, D (+) glucose, or D (−) levulose

To return to amino acids, the question as to which of the optical pairs is utilized by the body may now be answered In the case of each amino acid (except the inactive glycine, of course), the levo amino acid occurs in proteins never (or almost never) the dextro

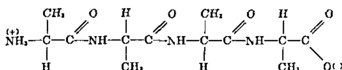
Levo-amino acids and protein structure. The significance of this universal occurrence of L amino acids in connection with protein structure is not immediately obvious The importance becomes apparent, however, when the spatial relationships of peptides are considered Let us take the case of two tetrapeptides one made up of four molecules of L-alanine and one made up of two molecules of L alanine and two of D alanine alternately placed First the case of the four L-alanines (formula XVIII) Despite appearances, the four molecules are all L-alanine Although the second and

fourth appear to be different from the first and third, it is only that they are viewed from the back and upside down If this is not obvious to the student from a consideration of the diagrams above models will make it so It might appear arbitrary to turn alternate molecules backwards and up side down, but again that is the fault of the inadequacy of the plane to picture solids If models were used instead, it would become apparent that only so could we place amino groups and carboxyl groups together so that a natural unstrained tetrahedral bond can be formed between them With three molecules of water split out the resulting peptide is as shown in formula XIX

The formula is not an accurate picture even according to the symbolism we have ourselves adopted Actually the carbon and nitrogen atoms in the horizontal chain are alternately above and below the plane of the



XVIII



XIX L-alanyl L-alanyl L-alanyl L-alanine

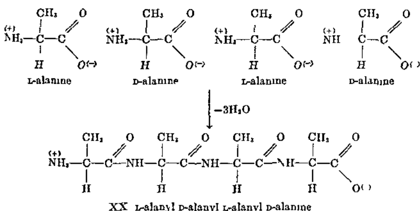
paper, while the hydrogen and methyl side chains are in a plane at a slight angle to the paper It is unnecessary to introduce those complications, however, since at the moment the point which should be brought out is that the methyl side chains occur alternately above and below the peptide backbone

In co ... ne formed by alter
nate L- ... ie final
result i ... all in
the same plane That of the second and fourth is slightly tilted to that in which the first and third are placed Essentially however, they may be looked upon as being on the same side of the peptide backbone

Is this difference between the appearance of the two types of peptide chains presented here important? It is If the two peptide chains here presented were built with models of atoms designed to represent actual atomic sizes proportionally (these are quite expensive) it would be seen that the D L-peptide with the methyl groups on the same side of the back

bone is comparatively crowded although the methyl group is the smallest side chain possessed by any amino acid other than glycine Where amino acids such as tryptophane or tyrosine are considered the extra space made available by the alternate placement of residues in the all L peptide is most useful It should be pointed out that very elaborate theories of protein structure, representing much labor and ingenuity, have eventually come to grief because of lack of room for side chains *even when placed alternately* (21)

There is, of course, no magic about the levo configuration A peptide chain containing all dextro-molecules would be as roomy as its levo sister Perhaps proteins began as levo polymers through chance Once established however, there would be no choice but to continue on the path chosen



XX L-alanyl D-alanyl L-alanyl D-alanine

Dextro-amino acids. The D amino acids are sometimes termed "un natural" amino acids but this is a rather unfortunate term, representing an unwarranted intrusion of emotionalism into science It is like speaking of 'improper fractions' and 'imaginary numbers" Actually, D amino acids *are* natural in the sense that they *do* occur, albeit rarely

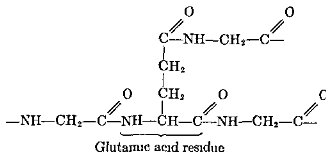
A D alanine occurs in the growth factor pantothenic acid (see Chapter 19), but is not the enantiomorph of the L-alanine occurring in proteins, since in it the amino group is attached to the β rather than the α carbon

In gramicidin S D phenylalanine occurs and this time it *is* the enantio morph of the 'natural phenylalanine Structurally, the formula of grami cidin S can be pictured (28) as in formula XXI

Since D amino acids have also been found in several antibiotics other than gramicidin S the thought has naturally arisen that in some way it is these "unnatural" molecules which are poisonous to bacteria To test this hypothesis tri and pentapeptides containing D phenylalanine and other amino acids of the type and in the order that they appear in the gramicidin

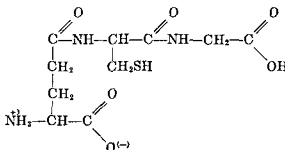
not yet been entirely determined, but important and illuminating advances have been made

Subsidiary covalent links. Perhaps the first variation on the peptide chain theme that might occur to the student is the possibility of branched chains One amino acid, lysine, possesses a second amino group, and two, aspartic acid and glutamic acid, possess each an additional carboxyl group It would seem reasonable that under certain conditions these additional



Glutamic acid residue

XXII Branched chain peptide



XXIII Glutathione

groups could form parts of new peptide chains branching off from the main backbone (formula XXII)

In living organisms, there is in fact an important tripeptide known as glutathione (formula XXIII) which in a simple way hints at such a possibility One of the residues is glutamic acid and it is combined in peptide [illegible] Small numbers

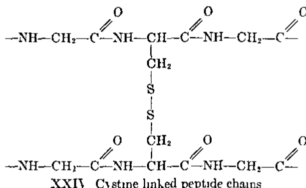
gation, a considerable [illegible]

bacillus (14)

However, *such branched chains* would not help explain the fact of protein instability The bonds involved would still be peptide in nature, with all the stability and comparative inertness to be expected of them Further

more, titration studies indicate that side chain amino and carboxyl groups are not involved in chain branching in most proteins

Another form of branching and one with more important consequences arises as a result of the chemical nature of the amino acid cystine Since it possesses a second amino group *and* a second carboxyl group, peptide chains can be conceived as branching off in two directions at once The net result would be to have two peptide chains linked together by means of a disulfide bond (formula XXIV) This is quite different from the types of branching discussed immediately above since the disulfide bond can be split by methods that would leave the peptide bond untouched Thus, under the influence of reducing agents, it is split to form two sulfhydryl (or thiol) groups



XXIV Cystine linked peptide chains

Proteins are indeed known in which adjacent peptide chains are held together by disulfide links in just the fashion described The keratins of hair, wool, and feathers are rich in cystine residues (hence, their characteristic odor when burnt) which link chains together, forming a three dimensional network that in great part accounts for the tensile strength of hair The breaking of such inter-chain linkages under conditions of moist heat, the consequent sliding of peptide chains past one another, and their hardening into a new mold by re formation of disulfide bonds upon cooling is the basis of the "permanent wave" Similarly, the four peptide chains of insulin, which have been discussed above are held together in the intact molecule by six disulfide links

A great many types of protein molecule, when denatured (subjected to heat or to other environmental conditions that cause them to lose their specific physiological properties) seem to possess sulfhydryl groups that could not be detected prior to denaturation While the source of such sulfhydryl groups is still disputable, one hypothesis is that denaturation

involves the breaking of disulfide links that served to hold the protein together in a specific fashion Since denaturation of a protein does not *usually* involve a change in its molecular weight, one may visualize a single polypeptide chain, coiled and looped into an intricate design, held in place by the periodic cystine residues Denaturation would then be a breaking *of the disulfides and a consequent uncoiling Unfortunately*, while the picture drawn is attractive, it is oversimplified On the one hand, many proteins do not form sulfhydryl groups on denaturation On the other, denaturation can be accomplished by methods so *mild that it is difficult to see how they would suffice to break disulfide linkages* Still other types of bondings have evidently yet to be considered

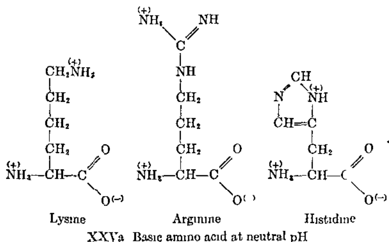
Electrovalent links. The variations of structure among the twenty odd commonly occurring amino acids are such as to allow considerable flexibility as far as possible types of interlinkages are concerned So far only covalent bonds have been considered, but the possibilities of electrovalences can not be ignored Of the various amino acids, the side chains of two (aspartic acid and glutamic acid) are sufficiently acidic so that at neutral pH they would exist largely as negatively charged ions Similarly, the side chains *of three amino acids (lysine, arginine, and histidine) are quite* basic and at neutral pH would exist as positively charged ions (formulas XXVa and XXVb)

Two peptide chains or two regions of a single peptide chain can thus be conceived as being held together by the mutal attractions of these positively and negatively charged ions Bonds so formed would be quite sensitive to changes in the pH and in the ionic strength of the medium in which proteins are dissolved Indications are not lacking that in certain specialized instances some protein properties may be explained in just this manner Insulin (to use it once again as an illustration) exists in solution as a complex of protein molecules It is built up of three (sometimes four) *identical* [illegible] composed as

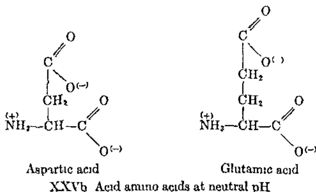
mild conditions without loss of physiological properties would seem to indicate the presence of such linkages between sub molecules there as well

Some chemists have proposed that it is such bonds which play the major role in keeping the protein molecule fixed in a given *pattern or configuration* and that the breaking of such linkages is what we call denaturation Again, this is perhaps an oversimplification since many proteins are quite stable over a *considerable range* of pH

Hydrogen bonds. Modern electronic theory of inter atomic bonding has completely disposed of the viewpoint that valence is a discontinuous phenomenon, that an atom can have a valence of one, two three, or four with nothing in between so that a valence of one and a half, for instance, is a ridiculous and meaningless concept The current attitude is to view



XXVa Basic amino acid at neutral pH



XXVb Acid amino acids at neutral pH

atoms as being held together by electronic interaction, the strength of which is dependent upon the make up of the molecule as a whole

Thus, the benzene molecule, which is ordinarily represented as possessing alternate single and double bonds, may more accurately be considered as having six carbon atoms held to one another by "one and a half" bonds According to this view each carbon atom in benzene has three ordinary valence bonds and a fourth which can be looked upon as having been broken into two ' half bonds" and each half used for one of the neighboring carbons

This modern concept of fractional valences is particularly illuminating when applied to the hydrogen atom The properties of many hydrogen containing compounds can be explained only on the assumption that hydrogen shares its single valence between two atoms, thus forming a link between those atoms known as 'hydrogen bond"

Not all atoms can take part in hydrogen bonding The only atoms, in fact which can do so to any significant extent are those of fluorine, oxygen, and nitrogen in order of diminishing ability Hydrogen containing compounds of these elements are in some ways unique, as may be exemplified by the most important of all such compounds, water Water is commonly looked upon as being simply H_2O Actually, it is more complicated than that because of hydrogen bonding Each water molecule "shares" its two hydrogens with two other molecules so that in the end each oxygen atom is surrounded, not by two but by *four* hydrogen atoms, much as a carbon atom is This type of bonding, while not very strong as compared with ordinary covalent links, serves to hold neighboring water molecules together and to impart an abnormal stability to the liquid and solid states (In steam, water molecules are too far apart and too energetic in their thermal motion to be able to form hydrogen bonds)

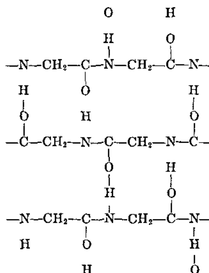
Thus, ice is abnormally difficult to melt, and water abnormally difficult to boil since in passing first from solid to liquid and then from liquid to vapor, it is necessary to break these hydrogen bonds in addition to over coming the normal cohesive forces among neutral atoms This can best be shown by comparing the melting and boiling points of H_2O with those of H_2S Ordinarily, among similar compounds, those with a higher molecular weight show higher melting and boiling points However, although H_2S has a molecular weight of 34, as compared with 18 for H_2O, it has a melting point of -82 9°C and a boiling point of -59 6°C , as compared with the well known 0°C and 100°C for the latter compound

In similar fashion, ammonia (NH_3) has a melting and a boiling point higher than those of phosphine (PH_3), and hydrogen fluoride (HF) bears the same anomalous relationship to the heavier hydrogen chloride (HCl) Again, if one of the hydrogens of water is replaced by a methyl group, the increase in molecular weight is more than compensated for by the loss of half the hydrogen bond forming capacity The boiling point of methyl alcohol (CH_3OH) is, therefore, only 64 7°C If the second hydrogen atom is also replaced by a methyl group, all possibilities for hydrogen bonding disappear and the boiling point of dimethyl ether (CH_3OCH_3) is -23 7°C

In this manner, the hydrogen bond can be shown to have a very real existence and the question arises as to whether the inner structure of the protein molecule depends upon the formation of hydrogen bonds between

various portions of the polypeptide chain (3) The following amino acids contain oxygen bound hydrogen in their side chains serine threonine tyrosine, hydroxyproline aspartic acid and glutamic acid The following contain nitrogen bound hydrogen tryptophane, histidine arginine and lysine All ten of these amino acids are theoretically capable of forming hydrogen bonds among themselves In addition, the peptide chain back bone itself is capable of forming hydrogen bonds with other such backbones as shown in formula XXVI

Mirsky and Pauling (19) in an interesting paper discuss denaturation as



XXVI Hydrogen bonds (—H—) and protein structure

a phenomenon involving the breakage of hydrogen bonds While their theory has not been universally accepted largely because of thermodynamic considerations, the details of which are beyond the scope of this book, it is a most attractive one since the mild conditions sufficing to denature proteins are just those which would be expected to break the very weak hydrogen bond while leaving the comparatively strong electrovalent and covalent bonds relatively untouched

It is possible now to summarize the methods by which polypeptide chains can be held together Table 3 lists the various types of bonds discussed above and the amino acids which may be involved in each case It will be noticed that eight of the common amino acids are not mentioned anywhere in the table They are glycine, alanine, valine, leucine, isoleucine,

phenylalanine, proline and methionine All of these but methionine are amino acids containing side chains which are purely hydrocarbon in nature Their particular functions with regard to protein structure are somewhat obscure It may be that the necessity or their presence in such chemical variety is in order that the varying sizes of their side chains may partly dictate the manner of folding of the polypeptide backbone For instance, there has been a suggestion (22) that the presence of a series of glycine residues *somewhere along the chain would result in a region where there* would be a complete absence of side chains with a consequent increase in chain flexibility These glycine *areas would thus be natural folding points* for the chain

Again, proline and hydroxyproline present unusual features as far as

TABLE 3

Side chain bond potentialities in protein structure

COVALENT BONDS	ELECTROVALENT BONDS	HYDROGEN BONDS
Glutamic acid	Glutamic acid	Glutamic acid
Aspartic acid	Aspartic acid	Aspartic acid
Lysine	Lysine	Lysine
Cystine	Histidine	Histidine
Cysteine	*Arginine*	*Arginine*
		Tryptophane
		Tyrosine
		Serine
		Threonine
		Hydroxyproline

amino acids are concerned Actually they contain not the —NH_2 group but an —NH— group Consequently, as part of the polypeptide backbone they alone among the amino acids possess a nitrogen atom to which is attached not *a single hydrocarbon and which therefore is completely in*capable of forming a hydrogen bond One could speculate, therefore, that regions of the polypeptide backbone which were particularly rich in proline would be relatively non sticky , or in other words would have less tendency to form subsidiary linkages with other polypeptide backbones

Denaturation

In the past few pages there *has been occasion several times to speak of* protein denaturation and from the statements made concerning it, the student may well have a fairly correct notion of what is meant by the term We will now treat it in more formal fashion

The term denaturation is a poor one and in itself merely a confession of *ignorance* A denatured protein is, if the word is accepted literally, a

protein that is no longer exactly as it originally existed in nature It is a negative word that tells us nothing and the best definitions that can be formulated for it are negative ones

It has been defined (23) as any non proteolytic modification of the unique structure of native proteins giving rise to definite changes in chemical physical or biological properties By Neurath s definition denaturation changes may be looked upon as those which do not involve actual breaking of the polypeptide backbone by proteolysis either enzymatic or chemical) yet the effect of energetic radiation such as ultra violet light or x rays is believed to involve among other things the breaking of that backbone Certainly radiation effects can not be excluded from consideration in any discussion of denaturation

Note also the phrase giving rise to definite changes in chemical physical or biological properties That is broad enough to take in everything The most apparent effect of extreme denaturation on protein is its loss of solubility in water and dilute salts and its tendency to coagulate irreversibly It is this which happens when eggs are boiled In that sense practical knowledge of denaturation and its effects is as old as the art of cooking There are more subtle chemical changes which can not be detected by the eye There are the liberations of sulfhydryl groups not present in the native proteins Their appearance is due either to the reductive cleavage of cystine in the process of denaturation or to an unfolding of the original protein molecule which would thus expose the sulfhydryl groups to the action of the chemical reagents used to detect them When proteins are dissolved in urea or in guanidine solutions a dissociation into smaller sub molecules frequently takes place (notably in the case of hemoglobin) This process can be reversed by the removal of the urea or guanidine by dialysis

There are biological changes in protein molecules which are symptomatic of denaturation and which may precede any detectable physical or chemical variations There are proteins which in extremely minute quantities display very characteristic and detectable properties Examples of these are viruses and enzymes Even when every effort is made to preserve them in their native states losses in their specific activity may be noted in the absence of any other detectable change

There are changes that can take place in protein molecules within the body and under the ordinary course of nature which would come under the heading of Neurath s definition For instance a mutation probably takes place as the result of some change in the structure of the nucleoprotein of a gene This change makes itself evident only in the next generation but the variation in its properties can then be detected and so the process must be termed one of denaturation Mutations can be considered accidents

but many physiological changes are not *Hemoglobin shuttles between the* oxygenated and non oxygenated forms, the actomyosin of muscle is alternately in a contracted and extended state These are definite changes in *properties that can be detected In fact, unless proteins in a state of nature* are looked upon as hopelessly static, which they most certainly can not be, all proteins are in a continual process of denaturation and renaturation

There is no real reason to believe that any individual protein can possibly be in a "native" state once it is separated from the living organism of which it is a part There is reason to suspect that proteins bear definitely *organized relationships to one another within the cell* relationships that are perhaps far too fragile to be detectable by our techniques as yet To attempt to equate the properties of a separated protein in a test tube and *those of a protein as part of a cell may well be like trying to study the* economy of a modern industrialized nation by observing how high the individual citizens thereof can jump

This same distressing broadness of scope appears when we list the types of chemical and physical agents that can bring about denaturation These include heat, short wave radiation, ultra sonic waves pressure, various chemicals *high acidity, high alkalinity, electron bombardment, neutron* bombardment, gas water interface effects (as in bubbling or frothing), dilution with water freezing and in short anything which can affect the vast complexities *of protein structure which are it would seem specifically de-* signed to be affected by virtually anything

[illegible] has a very complex and specific inner arrangement [illegible] *broken, the situa*[illegible] *ful removal of one* or two blocks from the bottom of a house of blocks The structure may *remain standing, and the bonds may be reformed when the disruptive in*fluence has been removed Thus, there is evidence that mild heating or mild changes in pH when not too prolonged, can cause changes in protein *properties which can be reversed to normal* upon cooling or upon neutralization (18)

If too many bonds are broken so that a whole loop of the protein falls *out of place, the effect* is that of the removal of one block too many from the house of blocks The entire structure comes down rather suddenly and irreversibly (4) In such a case, the highly ordered protein molecule becomes disorganized Instead of all the proteins being virtually identical, all have succeeded in falling apart in different ways (just as no two heaps of blocks will be exactly alike even though resulting from the disruption of two identical structures) Such an increase of disorder upon denaturation ought, if the above picture is a correct one, to result in an increase in the thermodynamic property known as entropy The entropy of denaturation can be

determined indirectly by measuring such things as the heat evolved in the process and is indeed found to increase markedly

Viewed in this fashion the problem of denaturation is simply another aspect of that of protein structure Once the latter problem has been satis factorily elucidated the former will simply disappear In fact it may already be time to relegate the word denaturation to the fate met by terms such as protoplasm phlogiston , and luminiferous ether which be gan as cloaks for ignorance and uncertainty and ended in meaninglessness and oblivion

Protein Isomerism

We have spoken of proteins as possessing great flexibility because of their size and complicated structure There is a way of attempting to ex press this flexibility in numbers and that is to calculate the various pro teins that can be built up from the twenty odd amino acid that act as monomers It is obvious that every different arrangement of amino acids would represent a different protein with different properties It is also ob vious that the number of such proteins is limited and in view of the enor mous chemical intricacies of even a simple organism it is reasonable to wonder if the limit is large enough to allow for the complexities of life If it is not it would be a strong indication that all our theories of protein structure are wrong

It might be well to start with the very simple protein salmine Salmine is a protamine occurring in salmon sperm It presents a certain problem to biochemists It together with a nucleic acid (see Chapter 7) forms a large part of the sperm and therefore within themselves they must carry at least some of the genetic factors that go to organize a particular salmon And yet salmine is almost indecently simple for a protein It contains only 58 amino acid residues of which no less than 40 are the single amino acid arginine It has a molecular weight less than 8 000 Can such a simple molecule even in combination with nucleic acid contain sufficient com plexity within itself to possess the capacity for producing one particular salmon and not another for certainly no two salmon are alike?

Let us consider first only the arrangement of the 58 amino acid residues along the chain Any of the 58 residues can be the first in the chain There are 58 possibilities there For each of the 58 cases any one of the remaining 57 amino acid residues can form the second in the chain We now have 58×57 different possible chains If we continue down the chain in this manner we end up with a total number of possibilities equal to $58 \times 57 \times 56 \times 55$ and so on all the way down to $5 \times 4 \times 3 \times 2 \times 1$ A number of this sort is called in shorthand mathematical notation factorial 58 and is symbolized as 58!

But not all the amino acid residues are different There are 40 arginines for instance In any given salmine chain, the 40 arginines can be shifted among themselves in any conceivable way and it would still be the same salmine chain Since 40 items can be shuffled among themselves in any of 40! ways, (the reasoning is the same as that in the preceding paragraph), the number of possible salmine chains now becomes 58!/40! In addition to the 40 arginines, salmine contains 7 serines 4 prolines 3 glycines 2 valines, 1 isoleucine and 1 alanine Taking the remaining duplications into account the formula for the number of possible salmine chains becomes

$$\frac{58!}{40! \times 7! \times 4! \times 3! \times 2!}$$

The actual number can be worked out by multiplication division, and patience It turns out to be the truly astounding value of 2×10^{24} That is certainly greater than the number of salmon in the world It is undoubtedly greater than all the salmon that ever lived

A molecule such as β lactoglobulin, discussed at the beginning of the chapter has possibilities of isomerism that are incomparably greater With *370 individual amino acid residues of 20 different kinds, the number of* arrangements possible would be of the order of 10^{425} A number such as that is quite beyond comprehension If the universe were a cube one billion

ligible when compared to the number of isomers of β lactoglobulin if only *amino* acid arrangement is considered! Take into account the variations in the finer structure, the exact location of the inter chain linkages and even the larger number becomes insignificant

We need go no further to decide that there is enough complexity in the

—indeed almost inevitable—the fact that no two humans are ever exactly alike (not even identical twins), as are no two rabbits or salmon or amoebae There is in fact enough complexity in the protein molecule to make it

ever existed in the past or will exist in the probable future

REFERENCES

1 ARCHIBALD R M Enzymatic methods in amino acid analysis Ann New York Acad Sc, 47 181 185 1946

2 Astbury W T X ray stud es of protein structure Cold Spring Harbor Symp Quant Biol 2 15-27 1934
3 Astbury W T The hydrogen bond in protein structure Tr Faraday Soc 36 871 880 1940
4 Boyd G A and Ibbel J J Absolute reaction kinetics of tobacco mosaic virus and a proposed theory of denaturation J Physical & Colloid Chem 52 1146-1153 1948
5 Brand E Amino acid composition of simple proteins Ann New York Acad Sc 47 187 2 8 1946
6 Brown C J et al Further studies in synthetic polypeptides Nature 163 834-835 1949 Kotchalski E Poly-α amino acids Advances Protein Chem 6 123 185 1951
7 Consden R et al Qualitative analysis of proteins A partition chromatographic method using paper Biochem J 38 224-23? 1944
8 Craig L C Identification of small amounts of organic compounds by distribution studies II Separation by countercurrent d stribution J Biol Chem 155 519-534 1944
9 Edman P A method for the determination of the amino acid sequence in peptides Arch Biochem 22 475-476 1949
10 Eiger I Z and Dawson C R Spectrophotometric method for the determination of the protein content of mushroom tyros nase preparations Arch Biochem 21 181 193 1949
11 Fankuchen I X ray diffraction and protein structure Advances Prote n Chem 2 15-27 1945
12 Fromageot C and de Garilhe M P La compos tion du lysozyme en acides amines Biochim et Biophys Acta 4 509-517 1950
13 Harris J I and Work T S The synthes s of peptides related to Gramicidin S and the significance of optical configuration in antibiotic peptides Biochem J 46 196-199 58? 589 1950
14 Haurowitz F and Bursa F The linkage of glutamic acid in protein molecules Biochem J 44 509-512 1949
15 Holiday E R Spectrophotometry of proteins Biochem J 30 179?-1803 1936
16 Klotz I M and Griswold P Infrared spectra and the amide linkage in a native globular protein Science 109 309-310 1949
17 Miller J A Do tumor proteins conta n D amino aci ls? A review of the controversy Cancer Res 10 65-72 1950
18 Mirsky A L Protein denaturation Cold Spring Harbor Symp Quant Biol 6 150-163 1938
19 Mirsky A E and Pauling L On the structure of native denatured and coagulated proteins Proc Nat Acad Sc 22 439-447 1936
20 Moore S and Stein W H Chromatography of amino acids on starch columns J Biol Chem 178 53-91 1949
21 Neurath H Intramolecular folding of polypeptide chains in relation to protein structure J Physical Chem 44 296-305 1940
22 Neurath H The role of glycine in protein structure J Am Chem Soc 65 2039-2041 1943
23 Neurath H et al The chemistry of prote n denaturation Chem Rev 34 157 265 1944

24 SANGER, F Some chemical investigations on the structure of insulin Cold Spring Harbor Symp Quant Biol, 14: 153-161, 1950

24a SANGER, F AND TUPPY, H The amino acid sequence in the phenylalanyl chain of insulin, Biochem J 49: 463-490, 1951

25 SCHMIDT, C L A *The Chemistry of Amino Acids and Proteins*, pp 183-220 Springfield, Ill, Charles C Thomas, 1938

26 SHEMIN, D, AND FOSTER, G L The isotope dilution method of amino acid analysis Ann New York Acad Sc, 47: 119-134, 1946

27 SNELL, E E Microbiological methods in amino acid analysis Ann New York Acad Sc, 47: 161 179, 1946

28 SYNGE, R L M "Gramicidin S" Over all chemical characteristics and amino acid composition Biochem J, 39: 363-367, 1945

29 WILLIAMS, R T, AND SYNGE, R L M (eds) Partition chromatography Biochem Soc Symp, No 3 Cambridge, Eng, Cambridge University Press, 1950

TABLE 3a

Amino acid composition of proteins

AMINO ACID	SYMBOL USED	NUMBER PER MOLECULE	
		β Lactoglobulin	Lysozyme
Glycine	Gly	8	11
Alanine	Ala	29	10
Leucine	Leu	50	8
Isoleucine	Ileu	27	7
Tyrosine	Tyr	9	3
Serine	Ser	20	10
Aspartic acid	Asp	36	13
Glutamic acid	Glu	24	4
Glutamine	Glu NH_2	32	16
Phenylalanine	Phe	9	2
Lysine	Lys	33	6
Arginine	Arg	7	13
Histidine	His	4	1
Tryptophane	Try	4	6
Proline	Pro	15	—
Valine	Val	21	8
Methionine	Met	9	0-2
Threonine	Thr	21	7
Cysteine	CySH	4	—
½ Cystine	CyS—	8	10 or 12
Water	H_2O	4	—

CHAPTER 3

Tissue Chemistry

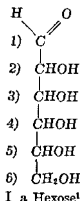
In the two preceding chapters the chemistry of proteins which form the fundamental living substratum of all tissues, has been discussed We are now ready to take up those biochemical characteristics of individual tissues which are of significance in the study of human metabolism Blood, a tissue which is unique both in its physical state since it is the only liquid tissue, and in many of its functions will be dealt with in a separate chapter Before proceeding to the individual tissues however, it would be well to recall a few aspects of the general chemistry of carbohydrates and lipids These two classes of compounds like the proteins, are universally distributed among living cells Their function is primarily, but not entirely that of serving as fuel for the body

CARBOHYDRATES

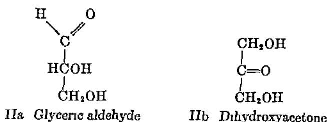
Carbohydrates have been defined as aldehydic or ketonic derivatives of polyhydric alcohols The names of the carbohydrates usually have the generic ending *ose* as for example, glucose Carbohydrates can be divided into groups depending upon the length of the carbon chain involved A carbohydrate containing six carbons is termed a *hexose* (formula I) By the same principle we have dioses, trioses, tetroses, pentoses, and so on The simplest carbohydrates of metabolic significance are the trioses, and two such are *glyceric aldehyde* (formula IIa) (or glycerose) and *dihydroxy acetone* (formula IIb) Note that the former is aldehydic and the latter ketonic For this reason glyceric aldehyde is referred to as an *aldotriose*, and dihydroxyacetone as a *ketotriose* Similar distinctions can be made with respect to carbohydrates with longer carbon chains Two very important carbohydrates glucose and fructose, are an aldohexose and keto hexose, respectively Carbohydrates containing aldehyde groups are spoken of collectively, regardless of the length of carbon chain, as *aldoses*, those with ketone groups as *ketoses* Among the ketoses, the ketone group is invariably located on the second carbon from the end

Aside from the ketone or aldehyde group each carbon atom in a carbohydrate is usually attached to a hydroxyl group Carbohydrates exist,

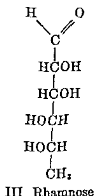
however, in which a hydroxyl group is missing along the carbon chain These may be named in two ways according to whether the hydroxyl group is missing from a carbon at the end of the chain or one not at the end An



I a Hexose[1]



IIa Glyceric aldehyde   *IIb* Dihydroxyacetone



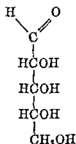
III Rhamnose

example of the former is rhamnose (formula *III*) Such a compound is usually referred to as a *methylpentose* or more generally still, a *methylose* An example where the missing hydroxyl group is not at the end may be taken from the two important pentoses which occur in nucleic acids (see Chapter 7) The fully hydroxylated pentose is named ribose (formula

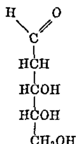
[1] The numbering system of carbohydrates begins at the aldehyde end (or at the end nearest the ketone group)

IVa), while the other is named desoxyribose (formula IVb) A compound such as the latter may be considered a desoxypentose, or more generally still a *desoxy-sugar*, or a desose Desoxy sugars in which more than one hydroxyl group is missing occur among the components of the digitalis glycosides

Carbohydrates which can be represented structurally as a single carbon chain (or as a single lactone ring) are termed monosaccharides The carbohydrates so far referred to in this chapter all belong to this class Carbohydrates which are composed of two monosaccharides linked by an oxygen bridge after removal of the elements of water are *disaccharides* Still more complicated compounds containing more than two such oxygen linked units also occur When the number of monosaccharide units is relatively few, such multiple compounds are *oligosaccharides*, and these include the disaccharides, trisaccharides, and so on Where the number of units is



IVa Ribose



IVb Desoxyribose

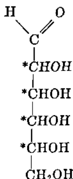
indefinite, or relatively large, the compound is termed a *polysaccharide* As the number of monosaccharide units becomes larger, the compounds gradually lose the characteristic monosaccharide properties of water solubility and sweetness Oligosaccharides retain these properties and are classified along with the monosaccharides under the general term, *sugars*

Monosaccharides

Optical activity Referring back to the formulas for glyceric aldehyde and dihydroxyacetone, earlier in this chapter, we notice that of the two it is glyceric aldehyde which possesses an asymmetric carbon In other words the center carbon of the three in glyceric aldehyde is surrounded by four different groups H, OH, CHO, and CH_2OH For this reason glyceric aldehyde possesses optical activity, while dihydroxyacetone, which does not possess an asymmetric carbon atom, does not In this respect it is glyceric aldehyde which is typical of the carbohydrates and dihydroxyacetone which is exceptional Although the principles of optical isomerism have been discussed in the previous chapter, certain further complexities arise in the study of carbohydrates since here for the first time we

faced with compounds which, as a class, contain more than one asymmetric carbon

Glucose, a typical hexose, has a formula which can be written as shown in formula V The four carbons marked by asterisks are asymmetric In each case, the exact spatial orientation of the hydrogen and the hydroxyl groups about the carbon is of significance in determining the optical properties of the compound For each asymmetric carbon there are two and only two ways of arranging the four groups about it If these two ways are denoted as *a* and *b*, then where two such carbons are found in a single compound four possibilities exist *aa ab*, *ba*, and *bb*, where three such are found, eight possibilities exist *aaa*, *aab*, *aba*, *baa*, *abb*, *bab*, *bba*, and *bbb* In general, where *n* asymmetric carbons exist in a compound, 2^n optical isomers may exist In the case of an aldohexose such as glucose, with four



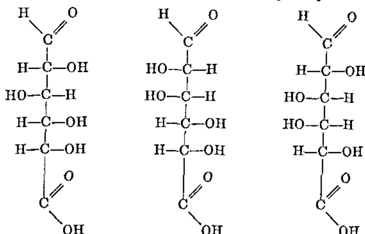
V Glucose (spatial considerations ignored)

asymmetric carbons, sixteen isomers may exist, of which only one is the sugar commonly termed glucose, or grape sugar And to confirm theory, sixteen aldohexoses have indeed been isolated or synthesized, and no more *than sixteen*

The structure of glucose, spatially, is conventionally represented as shown in formula VI Again, the exact spatial relationships are best followed by atom models since the molecule is not, as represented here, planar However by restricting efforts at superimposition to the plane of the paper, optical relationships can be adequately presented Thus, if glucose (properly termed D glucose (formula VIIa)) is compared with its mirror image, L glucose (formula VIIb), we see that the two compounds can not be superimposed Two other aldohexoses, D galactose and D mannose, have formulas as shown in formulas VIIc and VIId (The methods whereby the structure of the various sugars was determined are beyond the scope of this book, but are adequately discussed in advanced texts (23))

D glucose and L glucose are necessarily the same, but in opposite directions D galactose and D mannose both naturally have their own enantiomorphs in the compounds L-galactose and L mannose, the formulas of which the student should be able to write without further help The sixteen existing aldohexoses are, in fact, composed of eight pairs of enantiomorphs It should once again be emphasized that the prefixes D and L apply to the spatial structure of the compound only and not to the direction of optical rotations

Oxidized and reduced monosaccharides *Certain oxidized forms of the monosaccharides are of importance in human metabolism The uronic acids* are monosaccharide derivatives in which the primary alcohol group

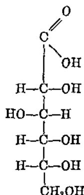


VIIIa D *Glucuronic* acid *VIIIb* D *Mannuronic* acid *VIIIc* D *Galactu*ronic acid

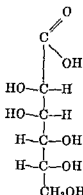
at the end of the chain furthest from the aldehyde is oxidized to a carboxyl *Uronic acids thus possess both aldehyde and carboxyl groups* Individual members of this group are named after the parent monosaccharide, as in glucuronic acid (formula VIIIa), galacturonic acid (formula VIIIb), and mannuronic acid (formula VIIIc) Uronic acids occur in the body as complex polysaccharides, usually in conjunction with amino sugars forming the prosthetic groups of the mucoproteins (see page 129)

In the *glyconic acids* it is the aldehyde carbon which is oxidized to carboxyl Such compounds are distinguished from the uronic acids in that they possess only the carboxyl group and not the aldehyde Again, individual glyconic acids derive their names from the parent compound, as gluconic acid (formula IXa), galactonic acid (formula IXc), and mannonic acid (formula IXb) Ascorbic acid is the lactone derivative of a dehydrogulonic acid, in turn derived from the aldohexose, gulose A still

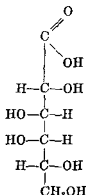
more complete oxidation of the monosaccharides results in derivatives in which both terminal carbons are oxidized to carboxyl groups These are termed the *saccharic acids* (formulas Xa and Xb)



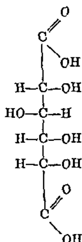
IXa D-Gluconic acid



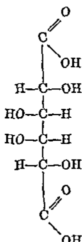
IXb D-Mannonic acid



IXc D-Galactonic acid



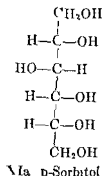
Xa D Saccharic acid (from glucose)

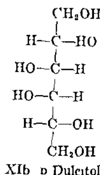


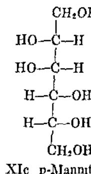
Xb D Mucic acid (from galactose)

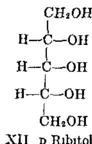
Reduction of the monosaccharides converts the aldehyde group to an alcohol, the resulting compound being a *sugar alcohol* Individual names are not always derived from the parent compound Thus the sugar alcohol of glucose is sorbitol (formula XIa) and that of galactose is dulcitol (formula XIb) That of mannose, on the other hand, is mannitol (formula XIc)

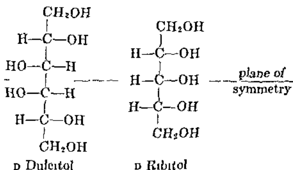
These sugar alcohols occur in various plants A five carbon sugar alco ribitol (formula XII) forms part of the vitamin riboflavin Dulcitol ribitol are compounds which display a type of optical activity we have


XIa D-Sorbitol


XIb D Dulcitol


XIc D-Mannit


XII D Ribitol



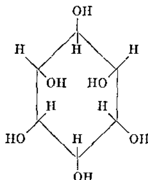
D Dulcitol D Ribitol
XIII

had occasion to consider hitherto If their formulas are inspected it will be seen that the former has four carbons and the latter three, which, considered individually, are asymmetric However, the compounds taken as a whole are symmetrical (formula XIII) This can be shown in two ways If an imaginary line is drawn perpendicular to the long axis of the molecule through the bond connecting carbons 3 and 4 in dulcitol, or through the

middle of carbon 3 in ribitol, the top half of the molecule in each case is the mirror image of the bottom half The lines drawn therefore represent lines of symmetry Secondly, if the mirror images of dulcitol or ribitol are constructed, they can be made to superimpose on the original molecule by a rotation of 180 degrees in the plane of the paper Dulcitol and ribitol thus do not have enantiomorphs

Compounds such as dulcitol and ribitol are termed *meso compounds* They are optically *inactive*, not because they lack asymmetric carbon atoms but because the asymmetric carbon atoms they do have may be divided into two groups which contribute equal and opposite asymmetries, the net effect being a perfect balance and zero optical activity

Inositols, sometimes called *cycloses*, are cyclic sugar alcohols (formula



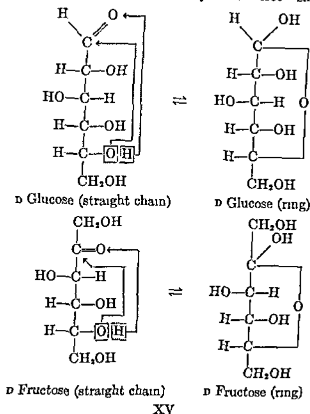
XIV *meso*-Inositol

XIV) which occur in significant concentration in the nervous system (see page 114)

Monosaccharide ring structure. Until now we have been considering monosaccharides as straight chain compounds They exist so only to a very small extent, however They are more accurately formulated as ring systems in which an oxygen bridge is formed between the aldehyde or ketone group and one of the other carbons, hydrogen being transferred to the aldehyde or ketone oxygen in the process (formula XV)

Theoretically, such an oxygen bridge can be formed between the aldehyde carbon and any of the others Actually, however, unions between carbons separated by two or three others, forming five and six membered rings, respectively, predominate This is because the angles between carbon valences are such that in forming a carbon chain, which is usually made to look straight when represented on paper, loops are formed in which a given carbon finds itself lying quite close to another carbon separated from it by three or four others This can be readily shown with the aid of atom models

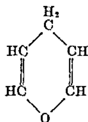
It is for this reason that five and six membered rings predominate among organic compounds They are relatively "strain free" since the valence



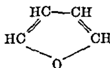
D Glucose (straight chain) D Glucose (ring)

D Fructose (straight chain) D Fructose (ring)

XV



XVIa Pyrane

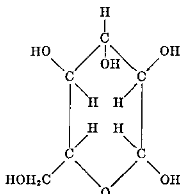


XVIb Furane

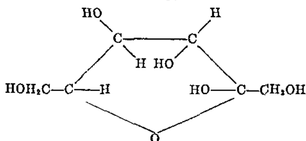
bonds need not be greatly distorted from their normal positions in their formation

Monosaccharides in the form of a six membered ring containing five carbons and one oxygen are spoken of as pyrane (formula XVIa) derivatives, since pyrane is the simplest compound containing such a ring system

imilarly, those consisting of five membered rings containing four carbons nd one oxygen are spoken of as furane derivatives (formula XVIb) These acts are included in the names given the rings formed Thus glucose exists rgely in the form of *glucopyranose* (formula XVIIa), and fructose in the orm of *fructofuranose* (formula XVIIb) Notice that in the formation of n oxygen bridge the aldehyde or ketone carbon becomes asymmetric Two



XVIIa Glucopyranose

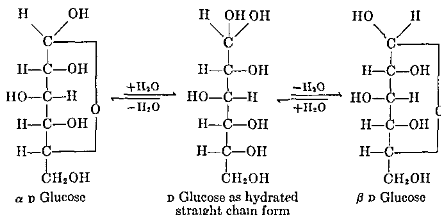


XVIIb Fructofuranose

additional optical isomers of glucose may be expected and are found They are termed alpha D glucopyranose and beta D glucopyranose, or more imply, alpha glucose and beta glucose Each of these forms has a different specific optical rotation (note that they are *not* enantiomorphs) However, a shift in orientation of the groups around carbon 1 takes place much more readily than is the case with any of the other carbons, probably through a process whereby the oxygen ring is alternately opened and closed In solution, therefore, an equilibrium between the alpha and beta forms of the sugar exists If pure alpha glucose or pure beta glucose is dissolved in water and its optical activity observed, the specific rotation

will be found to change steadily until in each case the same equilibrium figure is reached This process is known as *mutarotation* (formula XVIII)

It is sometimes more convenient to represent the ring structures of carbohydrates as pentagons or hexagons lying in a plane nearly perpendicular to the paper with the carbon bonds not involved in ring formation pointing up and down D Fructofuranose and the alpha and beta forms of D ribopyranose and D glucopyranose, as represented by such structures, are shown in formula XIX The student should be familiar with this kind of representation, since in any consideration of oligosaccharides such symbols are much more of a visual aid in understanding their structure than are the straight chain formulas more usually met

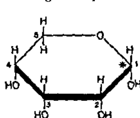


XVIII Mutarotation

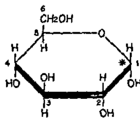
Glycosides The hydroxyl upon the carbon 1 of an alpha or beta aldose or the carbon 2 of a ketose can be replaced by an —OR group where R can represent any of a wide variety of organic groups Such compounds are known as *glycosides* and the sugar—O—R linkage as a *glycoside link* Glycosides involving particular sugars derive their names from the parent compound such as glucoside fructoside, or galactoside Both alpha and beta glycosides exist These are stable and do not exhibit mutarotation since the glycosides contain a relatively complex and immobile group in the place of the mobile hydrogen of the parent alpha or beta monosaccharide

If the glycoside linked group is not itself a sugar, it is called an *aglycone* In the glycosides of plants the sugar of most frequent occurrence is glucose In many cases, however, specific glycosides occur in which the sugar component is an unusual one, often occurring nowhere else in nature In the case of the digitalis glycosides, for instance, sugars with methoxy groups or lacking *two* hydroxyl groups occur Naturally occurring aglycones vary

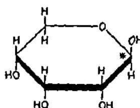
very widely in chemical nature, including phenols, alcohols, cyanide compounds, flavones, and steroids among others. Many glycosides are of great pharmacological importance. The digitalis group already mentioned is



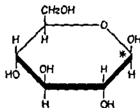
α-D-RIBOPYRANOSE



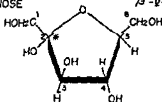
α-D-GLUCOPYRANOSE



β-D-RIBOPYRANOSE



β-D-GLUCOPYRANOSE



D-FRUCTOFURANOSE

XIX

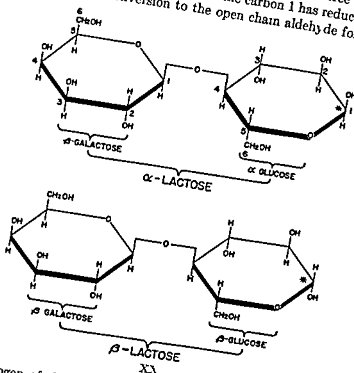
Note. The numbering system in the ring formulas is identical with that in the straight chain formulas, as will be evident on careful inspection. The asterisked carbon (carbon 1 in aldoses, carbon 2 in ketoses) is the carbon which, upon ring cleavage, becomes aldehydic or ketonic. The asterisked carbon is therefore known as the "reducing group." The reducing group is always adjacent to the ring oxygen and bears a hydroxyl group.

important in the treatment of cardiac disease. Phlorizin produces glycosuria on injection and phlorizinized dogs have been most useful in the study of carbohydrate metabolism.

Disaccharides

Two monosaccharides connected by a glycoside link constitute a disaccharide. The sugar found in milk is lactose, a disaccharide consisting of one

glucose unit and one galactose unit Note that the glycoside link is between the carbon 4 of glucose and the carbon 1 of galactose (formula XX) In other words, lactose is a glucose galactoside Furthermore, the glycoside link possesses the beta configuration so that the full chemical name of lactose is D glucopyranose-4 (beta D galactopyranoside) Note also that the carbon 1 of the glucose portion of lactose remains free This freedom leads to two results (a) the CHOH of the carbon 1 has reducing properties because of its easy conversion to the open chain aldehyde form by a shift

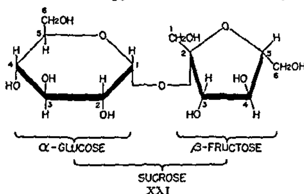


XX

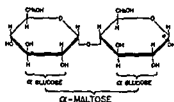
in the hydrogen of the hydroxyl group and the breaking of the oxygen bridge Lactose is thus a *reducing sugar* and exhibits mutarotation And, (b) it is conceivable that the free CHOH of the carbon 1 can be utilized to form still another glycoside link with a third monosaccharide

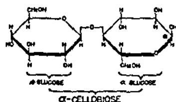
An example of a non reducing sugar is *sucrose*, the common sugar of the dinner table (formula XXI) It is a disaccharide of glucose and fructose, and is notable in that the glycoside forming carbons of both sugars, carbon 1 of the glucose and carbon 2 of the fructose are involved Sucrose can thus be looked upon either as a glucoside or a fructoside Since the alpha glycoside link of the glucose and the beta glycoside of the fructose are involved, the chemical name is alpha D glucopyranosyl beta D fructofurano

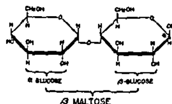
side The glycoside forming groups of both sugars are involved in the linkage so that no aldehyde can be formed through a break in either ring molecule Sucrose is therefore non reducing and does not display muta rotation Nor can further glycoside links be formed by it

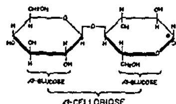


XXI









XXII

Note Both forms of maltose may be distinguished from both forms of cellobiose above since the lactone oxygens of both glucoses in maltose are on the same side of the rings while in cellobiose they are on the opposite side of the rings

The nature of the glycoside link, whether alpha or beta, is most important This is best demonstrated by the two disaccharides, *maltose* and *cellobiose* Both consist of two molecules of glucose, in which a carbon 1 of one glucose is linked via an oxygen atom to the carbon 4 of the other Maltose, however, is D glucopyranose-4 (alpha D glucopyranoside), while cellobiose is D glucopyranose-4 (beta D glucopyranoside) (formula XXII) The difference, as can be seen, lies in the orientation about the connecting

oxygen atom The two compounds can not be superimposed upon one another by turning one upside down or by twisting one ring through 180 degrees Physiologically the difference between the two is immense Maltose can be converted to glucose *in the gastro-intestinal tract through the* action of the enzyme maltase which acts specifically on the alpha glucoside linkage No enzyme exists in the human body which can act upon a beta glucoside linkage thus *cellobiose is nutritionally useless to man Both* maltose and cellobiose are reducing sugars capable of mutarotation and the formation of further glycoside links

Other disaccharides are known to occur in nature as well as trisaccharides and even a tetrasaccharide but none are nutritionally significant

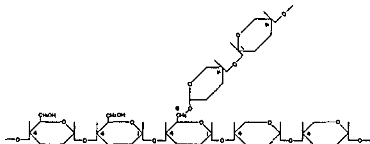
Polysaccharides

Multiple glycosides occur in which the number of monosaccharide units is large, ranging into the hundreds and even thousands These are the polysaccharides and examples are glycogen, starch, and cellulose Complete *hydrolysis of each of these yields only one monosaccharide component*, glucose Yet the difference between cellulose and starch is extreme, and this is due again to the manner in which glucose is interlinked in each case *In cellulose the glucoside linkages are all beta and in starch they are* alpha In other words any two adjacent glucose units in cellulose have the formula represented above for β cellobiose, while the corresponding disaccharide units in starch are α maltose Cellulose, like cellobiose, is nutritionally useless to man (except indirectly since it can be utilized by *herbivorous livestock through the action of their intestinal bacteria)*, while the starches as they occur in wheat, rice, potatoes, taro and manioc form the great nonprotein staples of the human diet

Starch is by no means to be considered a *well defined chemical species*, any more than protein may be so considered Starch molecules vary not only in the length of individual chains or in average length among species, but also in the nature of the chain itself One may visualize a starch molecule composed of a linear chain of glucose units Such a linear chain is *referred to as an amylose* molecule, and in it the carbon 1 of each glucose unit is connected by a glucoside link to the carbon 4 of its neighbor It is however possible to form a branched chain by connecting the carbon 1 of a glucose unit to the carbon 6 [illegible] *er already within an amylose* chain Such a branched molecule [illegible] to as an *amylopectin* and a portion of s[illegible] ule conta[illegible] of branching is here shown (formula V[illegible] ctins [illegible] themselves in the average straight [illegible] tween [illegible] in the degree of branching and *sub bra*[illegible] ylose [illegible] diffe[illegible] ays Thus, the

enzyme beta amylase will hydrolyze amylose completely to the disaccharide maltose Its action on amylopectin however, is limited to those end pieces beyond a branching point It can not catalyze the hydrolysis of the 1–6 linkage Again, iodine will oxidize amylose to form a characteristic, intensely blue black compound, while with amylopectin it yields a red product Most naturally occurring starches are mixtures of amylose and amylopectin In rare cases, as in certain varieties of maize rice, barley and other cereals usually referred to as waxy cereals starch can be found which is entirely amylopectin in nature

Upon hydrolysis, either acid or enzymatic, the starch chain is gradually broken into smaller and smaller fragments called *dextrins* As the average molecular weight grows smaller, the dextrins lose the typical starch prop



XXIII

erties of insolubility, coloration with iodine, and so on The change is a continuous one

Glycogen, otherwise known as animal starch, is a comparatively high molecular weight polysaccharide Carbohydrates in the animal body are stored in this form Chemically it resembles an amylopectin but is particularly highly branched, the interval between branches varying from as little as three glucose units to six or seven In ordinary plant amylopectins the corresponding intervals may extend up to 25 or 30 glucose units

Glucose is not the only monosaccharide capable of forming polysaccharides *Inulin*, a polysaccharide found in various plants consists of chains of fructose units Fructose polysaccharides other than inulin are known to exist as are polysaccharides consisting of galactose units or mannose units In general, such polysaccharides can be named by affixing the suffix *an* to the monosaccharide name Cellulose, amylose amylopectin and glycogen are examples of *glucosans*, and inulin is a *fructosan* Agar, so useful to bacteriologists, contains a *galactosan* Collectively, polysaccharides consisting of hexose units are *hexosans* *Pentosans*, such as *xylan* (consisting of xylose units), occur in woody plants along with cellulose

Polysaccharides consisting of more than one type of monosaccharide unit also occur Often these contain uronic acids, amino sugars, or sugar sulfates Hyaluronic acid, an important component of connective tissue, and heparin, an anticoagulant of clinical importance, are such mixed poly saccharides The polysaccharide prosthetic groups of the mucoproteins, the *mucopolysaccharides*, can also be included here

LIPIDS

Chemically speaking, lipids are far more diverse as a class than are the carbohydrates Actually, any organic substance capable of being metab olized by a living organism may be classified as a lipid if it is insoluble in water and soluble in such "fat solvents" as ether, chloroform, benzene, carbon tetrachloride, or carbon disulfide Three major subdivisions of lipids can be distinguished

1 *Simple lipids are esters which on hydrolysis yield only* alcohols and aliphatic monocarboxylic acids They contain only carbon, hydrogen, and oxygen

2 *Compound lipids* are esters which on hydrolysis yield molecules in addition to alcohols and aliphatic monocarboxylic acids Such extraneous molecules are most often nitrogen containing bases and phosphoric acid, and in some cases sulfuric acid or monosaccharides

3 *Associated lipids* are not esters but are found together with simple *and compound lipids in the early stages of most isolation procedures*, because of the similarity of their solubility properties

Along with these, a group known as the *derived lipids* is frequently spoken of, these being the fragments resulting from the hydrolysis of simple or compound lipids However, since many of these completely lack the typical lipid solubility properties, we prefer to discuss hydrolysis fragments along with the parent compounds, rather than to devote a separate section to them

Simple Lipids

The simple lipids vary in the nature both of the alcohol and the carboxylic *acids that make them up, but it is the* identity of the alcohol which is used as the basis of a further subclassification Thus, the simple lipids may be *divided into the fats and oils on one* hand, and the waxes on the

alcohol glycerol with three

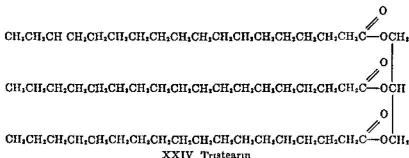
s with any

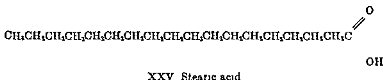
are usually

monohydroxy compounds The distinction between fats and oils is a purely physical one, the former name being given to those naturally occurring *mixtures of* glycerol esters which are solid under ordinary conditions,

while the latter is applied to those which are liquid This is a purely arbitrary distinction born of common usage, and chemically it is more satisfactory to apply the name *glyceride* to all glycerol esters, regardless of their melting points

Glycerides. The glycerides are the concentrated fuels of the body Where carbohydrate, in the form of glycogen, is the immediately available fuel (less than one day s supply being present in liver and muscle), glycerides can be stored, as circus sideshows often demonstrate practically indefinitely The greater economy of the glycerides as a fuel store when compared with carbohydrates is obvious from the fact that the former have a larger percentage of carbon and hydrogen and a lower percentage


XXIV Tristearin


XXV Stearic acid

of oxygen than the latter The empirical formula of glycogen for instance, is $(C_6H_{10}O_5)_n$, whereas that for tristearin, a typical glyceride, is $C_{57}H_{110}O_6$ A given weight of fat has over twice the fuel value of the same weight of carbohydrate

Tristearin (formula XXIV) consists of glycerine esterified with three molecules of stearic acid *Stearic acid* (formula XXV) belongs to the group of *fatty acids* a name usually applied to the aliphatic monocarboxylic acids, *because of their association with fats A variety of fatty acids have* been isolated from lipids but of these some half a dozen are most widespread and important, stearic acid being one of them Two closely related fatty acids of frequent occurrence are *palmitic acid* (formula XXVI) and *arachidic acid* (formula XXVII) Note that these three acids are very similar, differing only in the length of the hydrocarbon chain Palmitic acid contains sixteen carbons, stearic acid contains eighteen, and arachidic acid contains twenty

Fatty acids of this type containing still longer chains than arachidic or still shorter than palmitic are also found in nature, but in smaller quantity Particularly short chain fatty acids such as *butyric acid, caproic acid, caprylic acid,* and *capric acid,* which contain 4, 6, 8, and 10 carbons, are of interest since they occur in the milk fat of cows (hence in butter) and, to a lesser extent, in coconut and palm nut oils These short chain fatty acids are volatile and possess an offensive odor The odor of rancid butter is the result of liberation of butyric acid by hydrolysis Volatile fatty acids also contribute to the characteristic odors of certain cheeses and of perspiration

It will be noticed that the fatty acids so far mentioned all possess an even number of carbon atoms This is not because odd numbers of carbons render a fatty acid unstable Odd numbered fatty acids have been prepared synthetically and do not differ fundamentally from even numbered

$$CH_3CH_2CH_2\ CH_2CH_2CH_2CH_2CH_2\ CH_2CH_2CH_2CH_2CH_2CH_2CH_2C\begin{array}{l} /\!\!/ O \\ \diagdown OH \end{array}$$

XXVI Palmitic acid

$$CH_3CH_2CH_2CH_2\ CH_2CH_2CH_2\ CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2C\begin{array}{l} /\!\!/ O \\ \diagdown OH \end{array}$$

XXVII Arachidic acid

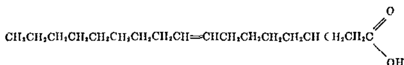
fatty acids in their chemical properties In the biosynthesis of fats however, the fundamental building stone is a two carbon fragment (see Chapter 12) which on being added to itself two or more times naturally yields even numbered acids only

In addition to the saturated fatty acids just mentioned certain unsaturated fatty acids are also of frequent natural occurrence Three such acids containing eighteen carbon atoms each and respectively, one, two and three double bonds are *oleic acid* (formula XXVIII) *linoleic acid* (formula XXIX), and *linolenic acid* (formula XXX) These differ from the saturated fatty acids most obviously in their lower melting points both as free acids and as glycerides Those fats which are generally liquid at room temperature and which are therefore termed oils usually contain considerable oleic acid

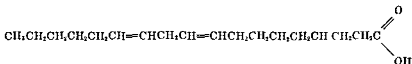
The unsaturated fatty acids are more reactive chemically than are the saturated fatty acids They will add on halogen atoms quite readily at the double bond, a fact which is important in fat analysis In addition they will autoxidize at the double bond, forming evil smelling peroxides which

contribute to the rancidity of glycerides If two or more double bonds are located in the molecule it can autoxidize polymerizing in the process, into a tough, insoluble amorphous material Linoleic acid and linolenic acid are found in considerable quantities in linseed oil, which because of this last mentioned polymerizing property is much used in paints

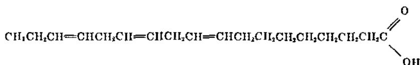
Other fatty acids are of limited occurrence Fish oils contain several fatty acids of very high degree of unsaturation In castor oil there is an important hydroxyl containing fatty acid *ricinoleic acid* (formula XXXI)



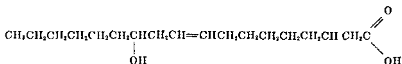
XXVIII Oleic acid



XXIX Linoleic acid



XXX Linolenic acid



XXXI Ricinoleic acid

which because of the hydroxyl group, differs from other fatty acids of like carbon content in being alcohol soluble For a full discussion of these and even rarer fatty acids, the student is referred to Hilditch (10)

Of all the fatty acids mentioned oleic acid palmitic acid, stearic acid and linoleic acid are by far the most commonly occurring Stearic acid occurs chiefly in animal glycerides and linoleic acid in the vegetable glycerides Oleic acid and palmitic acid occur nearly universally

While fatty acids do occur free in nature, they are much more commonly found esterified as glycerides or other lipids Fatty acids are examples of what we have referred to earlier as derived lipids since they are obtained from the naturally occurring glycerides by hydrolysis The trihydroxy

alcohol, glycerol, is in this sense also a derived lipid The glycerides are named according to the fatty acids that compose them The generic suffix for glycerides is *in* The glyceride containing three stearic acid groups has already been referred to as *tristearin* It is sometimes called simply *stearin* Similarly, we would have such glycerides as *tripalmitin*, *triolein* and *trilinolein* Glycerides such as these which contain only one kind of fatty acid are *simple glycerides*, while those containing more than one kind of fatty acid are *mixed glycerides* Examples of the latter (the names being self explanatory) are *stearodiolein*, *oleodipalmitin*, and *oleopalmitostearin* In the case of the mixed glycerides, isomerism is possible Where two fatty acids are present in a glyceride, as in oleodipalmitin, two isomers exist where three fatty acids are present, as in oleopalmitostearin, three arrangements exist

Mixed glycerides are of far more frequent occurrence in nature than are simple glycerides, and the tendency among organisms seems to be to avoid the formation of simple glycerides unless there is such a preponderance of a given fatty acid that the process becomes unavoidable In olive oil, for instance, 75 per cent of all the fatty acids is oleic acid and 50 per cent of the glyceride content is triolein

There is little to choose among the various glycerides as far as metabolic usefulness is concerned, and the differences in glyceride composition from species to species seem to depend most upon the temperature to which the glycerides are subjected It is important to the organism that its glyceride content be in the liquid state in order that it may be more readily subjected to the chemical reactions involved in its utilization Now the *melting point of a glyceride* decreases as the fatty acid constituents decrease in molecular weight or increase in their degree of unsaturation Thus tristearin has a melting point of 70 8°C, trilaurin (containing three molecules of the twelve carbon lauric acid) a melting point of 46 4°C, and triolein, one of −17°

fatty acids, therefor

the freezing point w

oil, for instance, melts at −5°C Warm blooded animals, whose glycerides are maintained constantly at temperatures in the neighborhood of 37°C, can utilize higher melting, more saturated compounds Lard, as an example, melts at about 34°C This adjustment of glyceride composition to environmental temperature can be found even within a given organism The glycerides of plants grown in a cooler climate are more unsaturated than those of similar plants grown in a warmer climate The glycerides in ox's hooves are more unsaturated than those elsewhere in the ox, and the glycerides in swine become more unsaturated as the skin is approached

The *chemical characterization of naturally occurring fats and oils* is not

easy in view of the fact that they usually consist of complex mixtures of glycerides which differ among themselves only slightly in their chemical properties It has therefore long been the custom to apply such tests to fats and oils as will yield average values for certain of their characteristics and in this way distinguish roughly among them

As an example, the grams of iodine which can be taken up by 100 grams of fat, is known as the *iodine value*, and is a measure of the average content of unsaturated fatty acids present, since it is only at the double bonds of those acids that iodine is taken up Thiocyanogen will add to only one double bond in linoleic acid and only two in linolenic acid The *thiocyanogen value* (grams of thiocyanogen taken up by 100 grams of fat) combined with the iodine value, would thus given an idea of the oleic acid content as differentiated from the combined linoleic and linolenic acid content The *acetyl value* is the milligrams of KOH required to neutralize the acetic acid from hydrolysis of one gram of acetylated fat Since only hydroxylated fatty acids can be acetylated the acetyl value is a measure of the average content of such acids Such values are of great use in characterizing and identifying fats and oils Linseed oil, for instance, would have a high iodine value, and castor oil a high acetyl value

The *saponification value* is the milligrams KOH required to hydrolyze one gram of fat This is a measure of the average molecular weight of the fatty acids present That this is so may not be obvious without reflection Each glyceride to be hydrolyzed requires the action of three molecules of KOH, one for each ester linkage, regardless of the length of the fatty acid chains in the molecule Where the average length of the fatty acid chain is comparatively small a given weight of fat will contain more glyceride molecules and therefore have a higher saponification value A comparatively low saponfication value would indicate the presence of long-chain fatty acids Specialized saponfication values, such as the *Reichert Meissl value* (milliliters N/10 KOH required to neutralize the steam-distillable acids from the hydrolysate of 5 grams of fat) are sometimes useful Only fatty acids with less than ten carbons are steam-distillable, and these occur appreciably only in butter and, to a lesser extent, in a few plant oils Butter thus has an appreciable Reichert Meissl value and can in this manner be easily distinguished from margarine

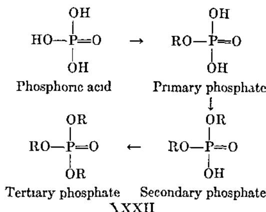
Waxes. Waxes have already been defined as esters of fatty acids with any alcohol other than glycerol For the most part these alcohols are long chain aliphatic monohydroxy compounds containing, usually, an even number of carbon atoms Such waxes are generally of little importance in human metabolism since they occur chiefly as insect secretions (beeswax, cochineal wax) or as protective coatings in plants (carnauba wax)

Waxes of greater importance to the human structure are esters of the

higher fatty acids with cholesterol, a secondary alcohol belonging to the class of sterols (a group which will be taken up in greater detail below) Cholesteryl esters, sometimes known as *cholesterides*, are found in the blood and form the major portion of the secretions of the sebaceous glands As sebum cholesterides fulfill the function of maintaining the gloss and flexibility of hair, and less usefully accumulate within the ear as ear wax *Lanolin*, used frequently in pharmaceutical and cosmetic preparations, is sheep sebum and owes its properties to its cholesteride content

Compound Lipids

Certain of the compound lipids represent the first substances of biochemical interest thus far discussed which contain phosphorus As our



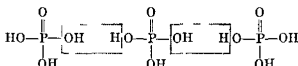
XXXII

study proceeds we will note how nearly universally phosphorus will intrude in one form or another in human growth and metabolism In living tissues phosphorus occurs invariably as phosphoric acid or as an ester or anhydride thereof Phosphoric acid contains three ionizable hydrogens (formula XXXII) Any or all of the hydrogens may be replaced by alkyl or acyl groups Organically substituted phosphates usually remain acid in character and are readily dissolved in basic solutions Two or even three molecules of phosphoric acid may condense with the elimination of water to form *pyrophosphate linkages* (formula XXXIII)

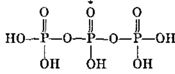
Phospholipids. Those compound lipids which yield phosphoric acid among their hydrolysis products are termed phospholipids These can be further subdivided according to the nature of the alcohols present as phosphoglycerides, phosphosphingosides, and phosphoinositides

Of these, the *phosphoglycerides* most closely resemble the simple lipids already discussed Complete hydrolysis of a molecule of *lecithin* the most common of the phosphoglycerides yields two molecules of fatty acids and

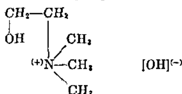
one each of glycerol, phosphoric acid, and choline *Choline* (formula XXXIV) is a highly basic nitrogen containing alcohol, the basicity being due to the fact that the nitrogen is fully methylated, and is consequently in the quaternary form Quaternary ammonium compounds, by virtue



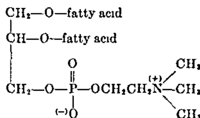




XXXIII Pyrophosphate links



XXXIV Choline



XXXV α-Lecithin

of the positive charge upon the nitrogen, act as strong bases (see page 233) A lecithin in which the phosphorylated choline is located on an end hydroxyl of the glycerol molecule is an alpha lecithin (formula XXXV) Where the phosphorylated choline is located on the middle carbon, the resulting compound is a beta lecithin (formula XXXVI) The nature of the fatty acids in lecithin is variable as well, and the net result is that tissue fractions to which the name ' lecithin" is applied are not chemical individuals but rather a complex mixture of closely related compounds

Frequently lumped together with the lecithins are the cephalins, which differ from the former in that the choline *is replaced by ethanolamine* (formula XXXVII) (also called colamine) Again, cephalins (formula XXXVIII) vary in the point of attachment of the phosphorylated base, and in the nature of the fatty acid content Cephalins, containing the amino acid serine in the place of ethanolamine, have been isolated from brain tissue and probably occur widely (1)

$$
\begin{array}{l}
CH_2\text{—O—fatty acid} \\
| \qquad\quad O \qquad\qquad\qquad\quad CH_3 \\
| \qquad\quad \| \qquad\qquad\quad (+) \diagup \\
CH\text{—O—}P\text{—}OCH_2CH_2N\text{—}CH_3 \\
| \qquad\quad | \qquad\qquad\qquad\quad \diagdown \\
| \qquad ^{(-)}O \qquad\qquad\qquad\quad CH_3 \\
| \\
CH_2\text{—O—fatty acid}
\end{array}
$$

XXXVI β Lecithin

$$
\begin{array}{ll}
CH_2\text{—} & CH_2 \\
| & | \\
OH & NH_2
\end{array}
$$

XXXVII Ethanolamine

$$
\begin{array}{l}
CH_2\text{—O—fatty acid} \\
| \\
CH\text{—O—fatty acid} \\
| \qquad\quad O \\
| \qquad\quad \| \\
CH_2O\text{—}P\text{—}OCH_2CH_2NH_2 \\
\qquad\quad | \\
\qquad\quad OH
\end{array}
$$

XXXVIII α Cephalin

$$
\begin{array}{l}
CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH{=}CHCHCHCH_2 \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \diagdown \quad \diagdown \quad \diagdown \\
\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad OH \; NH_2 \; OH
\end{array}
$$

XXXIX Sphingol

The *phosphosphingosides*, in addition to choline, phosphoric acid, and fatty acid, contain the complex amino alcohol, *sphingol* (formula XXXIX), or sphingosine The structure of these compounds is not as well established as is those of the phosphoglycerides The structure of *sphingomyelin* (formula XL) is the best known of the phosphosphingosides

The last of the phospholipid group, the *phosphoinositides*, are the most poorly characterized of all On hydrolysis *they yield inositol, a cyclic poly* hydroxy compound possessing properties reminiscent of the carbohydrates *In addition, phosphoric* acid, glycerol, fatty acids, carbohydrates, amino compounds, and even tartaric acid have been found among the hydrolysis

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH{=}CHCH(O-)CH(NH-\text{fatty acid})CH_2OH$

$-O-P(=O)(O^{(-)})-O-CH_2CH_2N^{(+)}(CH_3)_3$

XL Sphingomyelin

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2C(=O)OH$

XLI Lignoceric acid

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH(OH)C(=O)OH$

XLII Cerebronic acid

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH{=}CHCH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2C(=O)OH$

XLIII Nervonic acid

$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH{=}CHCH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH(OH)C(=O)OH$

XLIV Oxynervonic acid

products of various *phosphoinositides* The structure of no single such compound is yet completely known, however, although their importance is being increasingly appreciated

Galactolipids The most important of the compound lipids which do not contain phosphoric acid are the galactolipids These contain fatty acid, sphingol, and galactose An older name for the galactolipids, reflecting their occurrence in nerve tissue, is *cerebrosides* Four galactolipids are known differing among themselves in the nature of the fatty acid involved In one case, the acid is the normal 24 carbon lignoceric acid (formula XLI) In the other three cases (formulas *XLII, XLIII*, and *XLIV*), *cerebronic*, nervonic, and oxynervonic acids occur which are also 24 carbon acids, differing from lignoceric in the presence of a hydroxy group, a double bond, and both, respectively

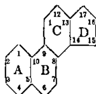
Solubility characteristics of the compound lipids The difference in the chemical structure of the compound lipids as compared with the simple lipids is reflected in their solubility behavior The simple lipids, *composed as they are of hydrocarbon chains primarily*, are non polar compounds (see Chapter 5) and therefore highly insoluble in water, or other polar solvents such as alcohol They are on the other hand, readily soluble in solvents which are themselves non polar, such as ether, chloro form or benzene The compound lipids, however, while containing hydro carbon chains in the form of fatty acids, sphingol, or both, contain in addition such highly polar fragments as phosphoric acid, choline, ethanol amine, inositol, galactose, or combinations thereof These polar fragments, while insufficient to convert the molecule as a whole into a water soluble substance, nevertheless exert a pronounced effect in that direction

Compound lipids, as a group, differ from simple lipids in being insoluble in acetone. Cephalin which is the least polar of the compound lipids, possess ing only a phosphate and an amino group in the molecule is ether soluble and alcohol insoluble Lecithin which is more polar in that it has the charged quaternary ion in the place of the amino group, while retaining ether solubility, becomes alcohol soluble as well Sphingomyelin, which in ad dition to a phosphate group and a quaternary ammonium ion possesses a second amino group is so polar as to be no longer soluble in ether These steps toward polarity on the part of the compound lipids with their con sequent effects on solubility are of great use in the fractionation of lipid mixtures, and are undoubtedly significant in the working of the human mechanism

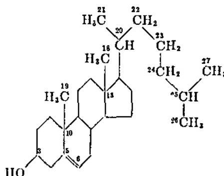
Associated Lipids

Those compounds which are not esters and yet are found in the lipid fraction of various tissues because of the similarity of their solubility prop

erties to those of simple and compound lipids have structures which are predominantly hydrocarbon in character Of these, the various steroids are the most important A *steroid* may be defined as an oxygen containing derivative of cyclopentanoperhydrophenanthrene (CPP) (formula XLV) Despite the apparent complexity of CPP such is the medical importance of its various derivatives that the student will do well to learn not only its structure but also the numbering system used to identify the individual carbons



XLV Cyclopentanoperhydrophenanthrene (CPP)

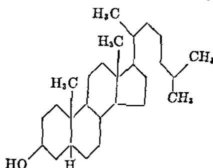


XLVI Cholesterol

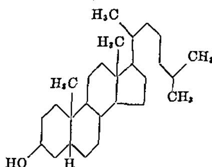
Sterols Sterols include those steroids which contain an OH group attached directly to the CPP nucleus and are otherwise hydrocarbon One such cholesterol (formula XLVI), has already been mentioned in the section on waxes Note here that the CPP nucleus is modified in three ways *(a) there is a double bond between carbons 5 and 6, (b) there is the* characteristic hydroxyl group of sterols on carbon 3, and, (c) there are hydrocarbon side chains on carbons 10, 13 and 17 Of the side chains, those on carbons 10 and 13 are methyl groups referred to because of their peculiar position on the angle between two rings as *angular methyls* These are characteristic of steroids in general and are absent only in a few cases The third side chain which is, in the case of cholesterol, an iso-octyl group shows considerably more variability among the different steroids The

numbering system of the CPP nucleus is extended to the carbons of the side chains in the manner shown in the cholesterol formula

Cholesterol is by far the most commonly occurring sterol in the human body It is accompanied to the extent of 2 per cent by its dihydro derivative beta cholestanol (formula XLVII) Another dihydro derivative coprosterol (formula XLVIII) occurs in the feces It will be noted that the formulas for these two compounds seem to differ only in that one bond is



XLVII β Cholestanol



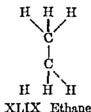
XLVIII Coprosterol

dotted in one case and solid in the other representing respectively bonds which are directed below and above the plane of the paper This form of isomerism has not been encountered previously in this book, and merits some discussion

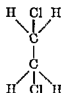
Cis trans isomerism Mention has already been made of the fact that

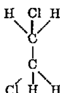
are located in a plane perpendicular to that in which the ... are found Under these circumstances a molecule of ethane may be represented as shown in formula XLIX—where the carbon-carbon bond is in the plane of the paper, the bold face bonds are visualized as projecting

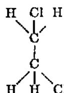
upwards from the paper and the dotted bonds projecting downwards into the paper If in such a model, one hydrogen on each carbon is replaced by a chlorine so that ethylene dichloride is formed, it will be seen that each chlorine can be placed on any one of three bonds, making a total of nine combinations Three of these are not superimposable if the molecule is viewed as a rigid unit (formula L), so that at least three varieties of ethylene dichloride should be distinguishable Actually only one kind of ethylene dichloride is known The reason for this is that free rotation is possible about the carbon carbon single bond By free rotation is meant that the amount of energy required to impart rotation to a group about a particular bond is so low that the kinetic energy derived from the normal thermal



XLIX Ethane







L Three non-superimposable forms of ethylene dichloride if the molecule is viewed as a rigid unit

motions of molecules at room temperature is sufficient If rotation about the carbon carbon single bond of ethylene dichloride is assumed, then each of the three "varieties" shown can be made to superimpose so that only one variety will in fact exist, which is in accord with experimental findings

Where rotation about a bond is restricted, however, cases of isomerism due to different arrangements of substituents become possible The energy required for rotation about a carbon-carbon double bond, for instance, is far more than can be acquired from thermal motions at room temperature The molecule of ethylene can be pictured as lying entirely in the plane of the paper, with the two CH_2 groups incapable of rotating out of it This rigidity means that two non superimposable forms of dichloroethylene can exist This form of isomerism is known as *cis trans isomerism*, the *cis* form being that in which the two substituents in question are on the same side of the molecule, and the *trans* form that in which they are on opposite sides (formulas LIa and LIb)

Cis trans isomerism differs from optical isomers in that *cis* and *trans* forms are not optically active, involve no asymmetric carbon atoms and are not mirror images of one another Unlike optical isomers, *cis trans* isomers usually, differ considerably in their chemical and physical properties The *trans* form is nearly always the more stable of the two, and usually has the higher melting and boiling point

An example of *cis trans* isomerism among substances of biochemical interest is found in the case of oleic acid (formula LII), which has the *cis*

H Cl
C
||
C
H Cl

LIa *cis* Dichloroethylene

H Cl
C
||
C
Cl H

LIb *trans*-Dichloroethylene

H H
C=C
$CH_3CH\cdot CH_2CH_2CH_2CH_2CH_2CH_2$ $CH_2CH_2CH_2CH_2CH_2CH_2CH_2C$ O OH

LII Oleic acid

H $CH_2CH_2CH_2CH_2CH_2CH_2CH_2C$ O OH
C=C
$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2$ H

LIII Elaidic acid

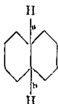
configuration The equivalent molecule in *trans* configuration, *elaidic acid* (formula LIII), does not occur in nature but can be formed from oleic acid by the action of nitrogen trioxide, N_2O_3, or by heating in the presence of selenium The differences between the two compounds are quite marked Whereas oleic acid melts at 14°C, elaidic acid has a melting point at 45°C Other naturally occurring unsaturated acids seem to posses the *cis* configuration at each of their double bonds

Cis trans isomerism is not restricted to compounds containing a double bond Rotation about a carbon carbon single bond may be restricted if that bond forms part of a ring In the case of decahydronaphthalene (decalin), for instance, two forms exist depending on the arrangement of the

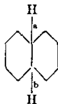
bonds about the carbon carbon single bond held in common by the two rings In *cis* decalin, the two bonds *a* and *b* both emerge on the same side of the plane of the paper, while in *trans* decalin they emerge on opposite sides (formulas LIVa and LIVb)

In beta cholestanol, rings A and B of the CPP nucleus are fused in the manner of *trans* decalin, while in coprosterol the fusion is in the manner of *cis*-decalin and that is the only difference between them Cholesterol, itself, is neither *cis* nor *trans* in this respect since the double bond between carbons 5 and 6 removes the possibility of a substituent hydrogen at carbon 5

In the case of the hydroxyl on carbon 3, the group may project either below or above the plane of the paper, the two isomers being distinguished arbitrarily as alpha *or beta hydroxy compounds Where the hydroxyl bond* is on the same side of the plane of the paper as the carbon 19 bond, it is



LIVa *cis* Decalin



LIVa *trans*-Decalin

arbitrarily assumed to lie above the plane and given the beta configuration Cholesterol and all other natural sterols belong in this group

The importance to the medical student of the phenomenon of *cis trans* isomerism lies *in the fact that the body can distinguish between such* isomers and use one in preference to the other Thus, while beta-cholestanol is found in tissues, its *cis* isomer, coprosterol, is not In steroids, other than the sterols stereoisomerism at other points—both at ring junctions and in the side chains—is also significant and for a fuller discussion of such problems the student is referred to a review article by Shoppee (25)

Bile acids and surface activity. The bile acids are steroids which in addition to one or more hydroxyl groups upon the CPP nucleus possess a side chain on carbon 17 which ends with a carboxyl group Such acids are found, as the name implies, in bile *cholic acid* occurring most abundantly Cholic acid (formula LV) possesses hydroxyl groups on carbons 3, 7, and 12 of which the hydroxyl group on carbon 3 is alpha in configuration, unlike those of the sterols Two other bile acids, found in smaller quantity in man are *desoxycholic acid* (formula LVI) and *chenodesoxycholic acid* (formula LVII) the *former possessing hydroxyls* on carbons 3 and 12, the latter on carbons 3 and 7

Cis trans isomerism differs from optical isomers in that *cis* and *trans* forms are not optically active, involve no asymmetric carbon atoms and are not mirror images of one another Unlike optical isomers *cis trans* isomers usually, differ considerably in their chemical and physical properties *The trans form is nearly always the more stable of the two, and usually has* the higher melting and boiling point

An example of *cis-trans isomerism among substances of* biochemical interest is found in the case of oleic acid (formula LII), which has the *cis*

$$\begin{array}{c} H \quad Cl \\ \diagdown \; \diagup \\ C \\ \| \\ C \\ \diagup \; \diagdown \\ H \quad Cl \end{array} \qquad\qquad \begin{array}{c} H \quad Cl \\ \diagdown \; \diagup \\ C \\ \| \\ C \\ \diagup \; \diagdown \\ Cl \quad H \end{array}$$

LIa *cis* Dichloroethylene - LIb *trans* Dichloroethylene

$$\begin{array}{ccc} & H \qquad\quad H & \\ & C{=}C & \\ CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2 & & CH_2CH_2CH_2CH_2CH_2CH_2CH_2C{\scriptstyle(=O)}OH \end{array}$$

LII Oleic acid

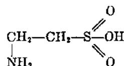
$$\begin{array}{ccc} & H \qquad\quad CH_2CH_2CH_2CH_2CH_2CH_2CH_2C{\scriptstyle(=O)}OH & \\ & C{=}C & \\ CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2 & \qquad\qquad H & \end{array}$$

LIII Elaidic acid

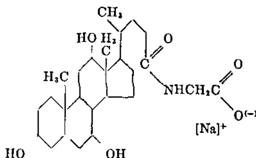
configuration The equivalent molecule in trans configuration elaidic acid (formula LIII), does not occur in nature but can be formed from oleic acid by the action of nitrogen trioxide, N_2O_3 or by heating in the presence of selenium The differences between the two compounds are quite marked Whereas oleic acid melts at 14°C, elaidic acid has a melting point at 45°C Other naturally occurring unsaturated acids seem to posses the *cis* configuration at each of their double bonds

Cis trans isomerism is not *restricted to compounds containing a double* bond Rotation about a carbon carbon single bond may be restricted if *that bond forms part of a ring* In the case of decahydronaphthalene (decalin), for instance, two forms exist depending on the arrangement of the

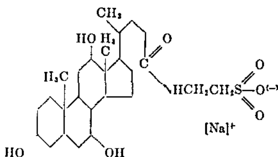
Bile salts are *surface active* since they possess polar and non polar groups In taurocholic acid, for instance, the CPP portion of the molecule, being largely hydrocarbon, is oil soluble and water insoluble The taurine portion is highly polar and consequently oil insoluble and water soluble If a small



LVIII Taurine



LIX Sodium glycocholate

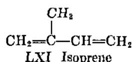


LX Sodium taurocholate

quantity of bile salt is added to water, the individual molecules will tend to orient themselves on the surface in such a way that a monomolecular film is formed in which the taurine (or glycine) portion is immersed in the water, while the CPP nucleus remains in the air Thus the surface tension of the system approaches that of CPP/air, which is much lower than the original value for water/air For this reason, extension of the area of water surface requires a considerably smaller input of energy in the presence of

bile salts than in their absence Thus, if water containing bile salt is shaken, a relatively stable foam is produced Other substances, notably the *soaps* (alkali salts of fatty acids), likewise possess surface activity and such compounds are referred to collectively as *detergents*

Actually, surface activity is but a special case of a more general phenomenon If bile salts are added to a system containing two phases, an aqueous and an oily one they tend to collect at the phase boundary Again the taurine or glycine portion of the molecule is oriented so as to dip into the water The CPP portion, on the other hand, extends into the oil Actually, the characteristic property of such substances is not merely surface activity, but rather "phase boundary activity" Here again, the interphase energy is lowered and extensions of its surface now require comparatively little energy Upon shaking such a system, an emulsion tends to form In such an emulsion a multitude of tiny oil droplets is suspended in the aqueous medium, each droplet being surrounded by a layer of properly oriented bile salt The possibility of a system composed of water droplets in an oily medium is not excluded The choice between the two possibilities



LXI Isoprene

rests on the relative quantity of each phase and on the nature of the surface-active agent It is by such emulsifying tendencies that ordinary soap, for instance, renders greasy or oily particles more "soluble" in water, and thus exhibits cleansing properties Similarly, bile salts aid the digestion and absorption of fats and fat soluble vitamins by their emulsification

Other steroids. A number of steroids found in the body are hormones These include the estrogens and androgens, which are sex hormones secreted by the female and male gonads respectively, progesterone which is secreted by the corpus luteum, and cortical steroids secreted by the adrenal cortex Their chemistry will be considered in Chapter 6 The chemistry of the D vitamins, which are related to steroids, will be considered in Chapter 19

Certain steroids which occur only in plants in the form of glycosides are of interest to the physician because of their pharmacological properties The chief of these occur among the active principles of digitalis

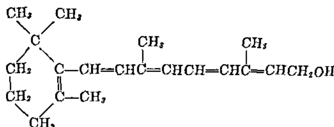
Carotenoids. Among the non steroid associated lipids are the carotenoids These are representatives of a much larger group of compounds, the isoprenoids, which are structurally related to isoprene (formula LXI) Carotenoids are those isoprenoids which are related to the hydrocarbon carotene ($C_{40}H_{56}$), which occurs as a mixture of two closely related substances—alpha- and beta carotene (formulas LXII and LXIII), plus a

LXII α-Carotene

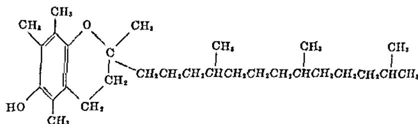
LXIII β-Carotene

LXIV γ-Carotene

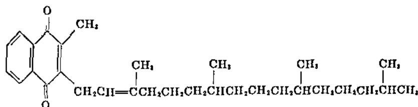
small amount of still a third isomer, gamma carotene (formula LXIV) The relationship of these compounds to isoprene is readily seen from a consideration of the formulas Carotene of dietary origin is found in the human body, and because of its intense color (due to its possession of numerous conjugated double bonds) it contributes notably to the pigmentation of skin, corpus luteum and blood serum



LXV Vitamin A



LXVI Vitamin E (α-tocopherol)



LXVII Vitamin K_1

Carotene is also the precursor of the A vitamins, the most common of which resembles structurally (formula LXV) a molecule of beta carotene broken in half with a hydroxyl group added to the broken end This and the other fat soluble vitamins, vitamins E and K (formulas LXVI and LXVII), both of which contain isoprenoid side chains, will be discussed in Chapter 19

OTHER UNIVERSAL CELL CONSTITUENTS

In addition to proteins, carbohydrates, and lipids, one other group of substances may be found in considerable quantities in all living cells

These are the *nucleic acids* usually found in conjunction with protein in both nucleus and cytoplasm Such is the importance of these substances that it is thought wise to devote an entire chapter (Chapter 7) to them They are phosphorus containing compounds, the molecules of which rival in size and complexity of structure the proteins themselves

Cells also contain a number of other substances in very low concentrations, yet indispensable to proper function These include the B vitamins, for instance, which will be considered individually in Chapter 19 The mineral constituents of cells are considered in Chapter 15

Lastly, as is perhaps needless to emphasize there is water In terms of sheer bulk it is the predominant constituent of the body constituting in the adult some 65 to 70 per cent of the body weight

CONNECTIVE AND SUPPORTING TISSUE

Connective and supporting tissue performs its functions through the possession of three types of substances of widely divergent nature (a) inorganic structures, (b) albuminoids, and (c) mucopolysaccharides and mucoproteins

Inorganic Components

The distinctive hardness and rigidity of bones and teeth are due to their inorganic content Although organic material is the continuous matrix of the bones and teeth that matrix is reinforced by inorganic salts The final inorganic content of bone at maturity is about 45 per cent Mineralization proceeds even further in the dentine and cement of teeth where about three fourths of the total is inorganic and reaches an extreme in enamel where some 98 per cent is inorganic The inorganic content of bones and teeth consists essentially of positive calcium ions and negative phosphate and carbonate ions, the phosphate/carbonate molar ratio being about 2 25 The whole is arranged in a crystal lattice similar to that of inorganic minerals known as apatites Details on the structure and quantitative ionic content of bones and teeth will be found in Chapter 15

Albuminoids

As a class, albuminoids are highly inert, either to solution or to digestion and possess the ability to form fibers of considerable toughness and tensile strength Usually they are composed of fewer kinds of amino acids than are the typical globular proteins, and the simpler amino acids predominate The most abundant such protein in connective tissue is *collagen* Fully one third of the organic matter of tendons consists of this protein It or proteins similar to it, may be found in cartilage, bone, and ligament as well, and in those portions of the teeth other than enamel Collagen, in terms of

amino acid content, is among the simpler proteins One third of its amino acid residues is glycine and another third is either proline or hydroxyproline Only small quantities of tyrosine are present while such amino acids as cystine, valine, and tryptophane are virtually absent, a fact which is of nutritional importance (see Chapter 10) X ray diffraction studies have indicated the possibility that collagen may consist of a succession of amino acids as follows proline (or hydroxyproline), glycine, another amino acid, proline, glycine, another, and so on indefinitely

Although collagen is an inert and tough material, it is converted in boiling water to *gelatin* Gelatin is a derived protein with no albuminoid properties Mustacchi (20), using x ray diffraction data, has come to the decision that the collagen fibril, or micelle, contains six polypeptide chains linked end to end Since it was already known that the fibril was thick enough to contain 4 to 6 polypeptide chains linked side by side, he concluded that each fibril contained a total of 24 to 36 polypeptide chains It would seem then that interpolypeptide linkages could be broken during boiling and that gelatin might consist of single polypeptide chains, rather than bundles thereof This increased simplicity of physical structure as compared with collagen probably explains the greater ease with which it is dissolved The exposure of new peptide linkages to easy attack by enzymes as a result of the break up of the collagen micelle would also explain the ready digestibility of gelatin by pepsin or trypsin as compared with the comparatively insignificant digestibility of collagen Gelatin is not coagulated by heat, withstanding boiling water without loss of any of its characteristic properties This is not an indication of any extraordinary resistance to denaturation, but rather a sign that gelatin is already as denatured as it can be

Elastin is another albuminoid of connective tissue, which resembles collagen in its properties It differs from collagen physically in that it is not converted to gelatin or a gelatin like substance on heating with boiling water Chemically, the amino acid distribution varies considerably Collagen is richer in amino acids with polar side chains such as arginine and

buminoids is by no means identical Collagen, considerably the more

high concentrations

d in

n a

and

2 per cent elastin (14), while the *ligamentum nuchae* of the ox is [illegible] per cent elastin and 17 per cent collagen

Mucopolysaccharides and Mucoproteins

The elastic fibrils of collagen and elastin are embedded in a matrix composed of mucoprotein—that is protein containing as a prosthetic group a mucopolysaccharide The terminology of this class of proteins has been somewhat confusing and Meyer (15) has suggested the following logical system

1 *Mucopolysaccharides* are polysaccharides containing a hexosamine as one component

2 *Mucoproteins* or *mucoids* are proteins containing carbohydrate as a prosthetic group where the hexosamine content is greater than 4 per cent of the whole molecule

3 *Glycoproteins* are proteins containing carbohydrate as a prosthetic group where the hexosamine content is less than 4 per cent of the whole molecule

According to these definitions glycoproteins would include many proteins

2-N ACETYLAMINO GALACTOSE SULFURIC ACID — GLUCURONIC ACID — GLUCURONIC ACID — 2-N ACETYLAMINO GALACTOSE SULFURIC ACID

LXVIII Repeating unit of chondroitin sulfuric acid

usually considered to be "simple" proteins Egg albumin serum albumin, and serum globulins would be included here This is an example of the inadequacies of the classical system of protein classification In actual practice, the terms mucoprotein mucoid, and glycoprotein are frequently used interchangeably

The mucoproteins that occur in the various connective and supporting tissues resemble one another closely although they are usually distinguished by such names as *tendomucoid, chondromucoid* and *osseomucoid* for the varieties that occur in tendons, cartilage, and bone, respectively All three possess as the prosthetic group *chondroitin sulfuric acid* containing acetyl galactosamine and glucuronic acid, connected by glycosidic links The galactosamine groups are further esterified with sulfuric acid The molecular weight of chondroitin sulfuric acid (formula LXVIII) has been placed between 200 000 and 400 000 A mucopolysaccharide very similar to chondroitin sulfuric acid is *mucoitin sulfuric acid* which differs from the former only in that the acetyl galactosamine group is replaced by an acetyl glucosamine This prosthetic group occurs in the mucoproteins of saliva and gastric juice

Most mucoproteins are characterized by high solubility in water and relative resistance towards denaturation The amount of polysaccharide content necessary to so protect a protein is the basis for the otherwise arbitrary *distinction of* Meyer's between mucoproteins and glycoproteins

Mucopolysaccharides in the free state are also found in connective tissue The chief of these is *hyaluronic acid*, which contains acetyl glucosamine and glucuronic acid Its composition differs from that of mucoitin sulfuric acid in the following ways (1) it is not found in combination with protein, (2) it does not contain sulfuric acid groups, (3) it has a higher molecular weight In solution hyaluronic *acid is exceedingly viscous and exhibits* marked double refraction of flow From these properties an average molecular weight of 500,000 has been calculated, but this is probably far too low a figure Hyaluronic acid has been isolated from umbilical cord, synovial *fluid skin, and some mesenchymal tumors In connective* tissue, its occurrence would seem to be established by the action of hyaluronidase and by histochemical staining techniques From such experiments it would seem that hyaluronic acid is widely distributed and forms at least part of the intercellular ground substance in general

Hyaluronidase is an enzyme capable of catalyzing the depolymerization of hyaluronic acid, or in other words, of breaking the glycoside bonds and forming end products of relatively small molecular weight It was originally called "spreading factor" because in its presence injected fluids seemed to permeate tissues at a more rapid rate It does this by causing the depolymerization and consequent loss of viscosity of the hyaluronic acid between cells and within connective tissue thus allowing substances to soak through more quickly Hyaluronidase may not be a chemical individual It is by no means entirely established that the bonds linking the saccharide units in hyaluronic acid are all glycosidic in nature Ester and anhydride linkages have been suggested and it is not beyond the bounds of possibility that spreading factor" may turn out to be a rather complicated system *of enzymes Hyaluronidase is thought to play a part in* facilitating entrance of spermatozoa into ova during fertilization (see Chapter 9) and is one of the factors involved in bacterial invasiveness

Lysozyme is an enzyme noted originally for its lytic effect upon certain bacteria Some of its sources are nasal mucus, tears leukocytes, and egg white Meyer and Hahnel (16) have shown the enzyme to be a mucopolysaccharase similar in its properties to hyaluronidase Presumably, it lyses bacteria through *its hydrolytic action* upon the bacterial capsule, which is usually a mucopolysaccharide Its role as a protective agent against bacterial invasion is, however, uncertain Most of the bacterial species on which its lytic activity has been demonstrated are non pathogenic

Heparin, which is a mucopolysaccharide produced in liver and lung, is

an anticoagulant and is used in maintaining blood in a fluid state for transfusion and to prevent thrombosis It is a mucoitin sulfuric acid in which, however, five sulfuric acid esters rather than two are present for each tetrasaccharide unit Naturally occurring substances of medical interest which have been found to be mucopolysaccharide in whole or in part include various pituitary hormones and the A B, and O blood group substances

SKIN AND ITS APPENDAGES

Skin, like connective tissue must be tough and flexible to fulfill its purpose It is even more roughly treated since it withstands directly the buffeting of the outside environment Like connective tissue it is rich in tough, inert albuminoids and in mucoproteins which act as an intercellular ground substance The albuminoid which is characteristic of skin is *keratin*

Keratin

Keratin possesses the properties of collagen to a greater extreme Like collagen it is insoluble in any solvent that does not attack it chemically, but it lacks even the limited digestibility of collagen Neither pepsin nor trypsin, both of which will slowly attack collagen, has any appreciable effect on keratin in its natural state This is not because of any fundamental difference between keratin and other proteins in terms of its chemical make up, but is rather due to the physical nature of the albuminoid If keratin is finely ground in a ball mill, not only does it become somewhat digestible but it even becomes appreciably soluble

Keratin is the characteristic protein not only of skin itself but of the various skin appendages such as hair, wool, nails, hooves, horns, and feathers What protein exists in the enamel of teeth is keratin although that in the dentine and cement is collagen A form of keratin exists in nerve tissue, a biochemical indication of ectodermal origin Keratin differs chemically from the albuminoids of connective tissue chiefly in its unusually high content of cystine, human hair containing 15 to 20 per cent of that amino acid It is the cystine content which imparts the "odor of burning feathers" to burning feathers skin, hair, or wool The presence of cystine helps explain the toughness and inertness of keratin It forms disulfide bridges (see Chapter 2) between adjacent polypeptide backbones, forming a three-dimensional polymer, in which the separate fibrils mutually strengthen one another

Keratin is also characterized by a relatively constant proportion of the basic amino acids histidine, lysine and arginine The ratio of these is 1 4 12 in the keratin of skin appendages In skin itself, the ratio is somewhat different and the two types of keratin are sometimes referred to

respectively as *eukeratin* and *pseudokeratin* Pseudokeratins contain only about half the percentage of cystine that eukeratins do, which is reasonable if one compares the toughness required of hair or nails with that required of skin itself

Skin Pigments

One of the most noticeable variations among human beings is that of *their external coloring*—*that is*, the extent of pigmentation of the hair, skin, and eyes These variations in pigmentation are not due to the possession *by one group of human beings of any pigment not possessed by others*, but rather to differences in the proportions of several pigments, each of which is possessed by all normal humans Of these, the most important by far is *melanin*

Melanin. This is a dark brown pigment, insoluble in all ordinary reagents but alkali and only slowly soluble in that It is a polyphenol polymer synthesized in the body from the amino acid tyrosine Melanin is present *in the epidermis of all men who are not albinos It is partly in the form of* complexes with protein which are referred to as *melanoproteins* In the classical protein classification these come under the heading of chromoproteins

The amount of melanin in the skin varies in a roughly direct manner with the average local intensity of solar ultraviolet radiation Scandinavians are generally "fair", that is, have but little melanin in the skin, while the inhabitants of west and central *Africa have* skin *rich in melanin and are* consequently dark skinned Inhabitants of the Mediterranean shores are intermediate Melanin acts as a radiation absorber and protects the body from the bad effects of excessive exposure to the ultraviolet light of the sun The positive survival value of a high melanin content of skin in hot, sunny climates is obvious The difference between a blond who "burns" at the seashore and a brunette who "tans" is familiar to all

Under stimulation of *sunlight, production of melanin is increased* and there is a general darkening of the skin, known as tanning Where the natural capacity for melanin production is deficient, the darkening is often not uniform but occurs spottily in the form of freckles There are local increases in melanin formation under the influences of various normal and pathological changes Examples are the darkening of the areolar area of the breast during pregnancy, and the increase in melanin in the malignant proliferation of pigment producing *cells* These last are known as *melanomas*, and ordinary moles are benign forms thereof

Melanin is also partly responsible for hair and eye color As its concentration is progressively diluted, hair may be black, brown, red, blond, and

white with innumerable shades between Eyes shade through the various intensities of brown to hazel and eventually blue

Individuals are occasionally born without the capacity to form melanin at all This is probably due to the absence of the enzyme *tyrosinase*, which catalyzes the first stage of the conversion of tyrosine to melanin Such individuals are called *albinos* and they may occur in families the members of which possess all possible degrees of pigmentation They are characterized by extremely fair skin white hair and unpigmented eyes

The amount of melanin in the skin in any degree has no known biochemical connection with the presence or absence of any other human characteristics

Other skin pigments The role of *carotene*, which is present in small percentages in skin lipid fractions is second in importance only to melanin Its presence imparts a yellow coloration to the skin Where melanin is present in large concentrations the effect is of course masked but among the melanin poor groups of humans differences in carotene content may be markedly visible to the naked eye Human groups in central and east Asia are relatively high in carotene pigmentation for instance while those of Europe are relatively low

The various pigments present in the blood make their contribution to apparent skin color Of these hemoglobin and oxyhemoglobin are most important They impart a ruddy color to the skin which is most noticeable in individuals poor in melanin and carotene In albinos, the color due to blood itself is the only significant pigmentation effect, so that the eyes for instance being devoid of melanin possess pink irides Unusual contributions to skin color occur in various pathological states There is the yellow green color in jaundice which is due to increased circulating bile pigment, and also pigmentation as the result of intake of chemicals accidental or otherwise as in the use of atabrine the chief antimalarial of World War II For a discussion of skin pigmentation as an aid to diagnosis see Jeghers (12)

Hair

Hair is keratin fiber in an almost pure state As such it has been much used in physical chemical investigation of protein structure Hair, in its ordinary state, is a cluster or bundle of partially folded polypeptide chains bound together by disulfide links Keratin in this state is known as *alpha keratin* When soaked in water hair can be stretched out to about twice its original length and x ray diffraction studies on stretched hair reveal that the polypeptide backbone has been extended to a simple zig zag pattern similar to that of silk fibroin In this state the protein is *beta*

keratin If hair is subjected to damp heat, or to alkaline solutions at room temperature the disulfide links are broken and the polypeptide chain can then fold up to an even greater extent than it normally does A super contracted form thus makes its appearance in which the hair shrinks to about 70 per cent of its original length

It is the last phenomenon which is made use of in permanent waving When hair is subjected to damp heat so that disulfide links are broken individual polypeptide chains hitherto bound together can slide past one another and take up new relative positions if the hair is artificially curled while in this state The use of an alkaline agent such as ammonia hastens the procedure If the hair is maintained in the curled position and allowed to dry disulfide links are again formed between neighboring chains holding them firmly in the new positions Permanent waving can be performed without the use of heat if the disulfide groups are broken by reduction to sulfhydryl groups So called cold waves make use of various reducing agents for this purpose including mercaptans thioglycolate and other such compounds The indiscriminate use of these substances is not entirely without its attendant dangers Toxic reactions and even temporary baldness may result from their misuse (13)

Ordinary baldness while not usually a pathological state is often considered a social handicap and those suffering from it are frequently quite disturbed about it and seek relief either within or without the borders of medicine Unfortunately non pathological baldness is not yet amenable to treatment and may never be There are at least three factors involved in baldness First there is age Hair generally decreases in quantity with age in both men and women Secondly there are genetic factors In some men the hair thins markedly during middle life and sometimes as early as in the twenties This is apparently a hereditary trait which can not be altered by external treatment by lotions and salves any more than the pigmentation of the eye can be changed by the use of eyewash Lastly loss of hair is apparently associated with the presence of androgens That is even in the presence of a gene for baldness hair will not fall out excessively if androgen concentration is comparatively low Eunuchs for instance (where castration has occurred before baldness has already appeared) practically never lose their hair It is through the effect of increased androgen concentration that baldness occurs in adults rather than in children and in males rather than in females

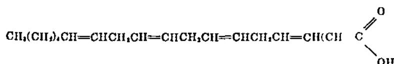
Dietary correctives for undesirable hair characteristics have been established for animals Thus addition of cystine to the diet increases the weight of wool grown by sheep in a given time Para aminobenzoic acid achieved some notoriety as a corrective for premature graying of hair in

rats Neither substance, unfortunately has as yet proved useful in the case of human beings

ADIPOSE TISSUE

The lipid content of the human body can be divided into two broad classes There are first the lipids of all tissue cells These are in the main compound lipids and sterols which are essential to the cell mechanism and are not appreciably consumed during periods of emaciation Secondly there is *depot fat*, which is composed of fat cells which are in essence merely fat droplets surrounded by a thin shell of protoplasm scarcely more than a membrane Such fat is almost entirely simple lipid in nature and its function is mainly that of serving as an energy store Depot fat decreases in quantity during periods of caloric restriction

About half of the depot fat is deposited in the subcutaneous regions Considerable quantities are located in the mesentery and about the kidneys In the latter case it serves to provide mechanical support and pro-



LXIX Arachidonic acid

tection Subcutaneous fat is poorly vascularized and is itself a poor heat conductor It therefore serves as insulation against cold The chemical nature of depot fat varies to a certain extent with the nature of the fat in the diet The subcutaneous fat of a dog fed on linseed oil remains liquid at 0°C as a result of the unusually large quantities of unsaturated acids incorporated therein Fed on mutton fat, the dog's adipose tissue becomes firmer and its melting point higher

Human fat under normal conditions, is liquid at blood temperature due to a considerable content of oleic acid, linoleic acid, and palmitoleic acid The three together account for about 60 per cent of the fatty acid content of adipose tissue The remaining fatty acid content is almost entirely saturated Palmitic acid is the most common saturated acid composing about 20 per cent of the total The presence of *arachidonic acid* in small concentration is to be noted Arachidonic acid (formula LXIX) is a highly unsaturated 20-carbon acid, containing four double bonds In rats, at least its absence in the diet has been shown to cause dermatitis The adrenals are particularly rich in arachidonic acid where it forms 22 per cent of the fatty acid content of their phospholipids

The composition of human depot fat as glycerides rather than as fatty

keratin If hair is subjected to damp heat, or to alkaline solutions at room temperature, the disulfide links are broken and the polypeptide chain can then fold up to an even greater extent than it normally does A "supercontracted" form thus makes its appearance, in which the hair shrinks to about 70 per cent of its original length

It is the last phenomenon which is made use of in "permanent waving' When hair is subjected to damp heat so that disulfide links are broken, individual polypeptide chains hitherto bound together can slide past one another and take up new relative positions if the hair is artificially curled while in this state The use of an alkaline agent such as ammonia hastens the procedure If the hair is maintained in the curled position and allowed to dry, disulfide links are again formed between neighboring chains, holding them firmly in the new positions Permanent waving can be performed without the use of heat if the disulfide groups are broken by reduction to sulfhydryl groups So called "cold waves" make use of various reducing agents for this purpose, including mercaptans, thioglycolate, and other such compounds The indiscriminate use of these substances is not entirely without its attendant dangers Toxic reactions and even temporary baldness may result from their misuse (13)

Ordinary baldness, while not usually a pathological state, is often considered a social handicap, and those suffering from it are frequently quite disturbed about it and seek relief either within or without the borders of medicine Unfortunately, non pathological baldness is not yet amenable to treatment and may never be There are at least three factors involved in baldness First, there is age Hair generally decreases in quantity with age in both men and women Secondly, there are genetic factors In some men, the hair thins markedly during middle life and sometimes as early as in the twenties This is apparently a hereditary trait which can not be altered by external treatment by lotions and salves any more than the pigmentation of the eye can be changed by the use of eyewash Lastly, loss of hair is apparently associated with the presence of androgens That is, even in the presence of a gene for baldness, hair will not fall out excessively if androgen concentration is comparatively low Eunuchs, for instance (where castration has occurred before baldness has already appeared), practically never lose their hair It is through the effect of increased androgen concentration that baldness occurs in adults rather than in children, and in males rather than in females

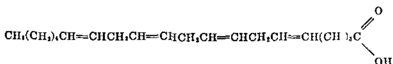
Dietary correctives for undesirable hair characteristics have been established for animals Thus, addition of cystine to the diet increases the weight of wool grown by sheep in a given time Para aminobenzoic acid achieved some notoriety as a corrective for premature graying of hair in

rats Neither substance, unfortunately has as yet proved useful in the case of human beings

ADIPOSE TISSUE

The lipid content of the human body can be divided into two broad classes There are first the lipids of all tissue cells These are in the main, compound lipids and sterols, which are essential to the cell mechanism and are not appreciably consumed during periods of emaciation Secondly, there is *depot fat*, which is composed of fat cells which are in essence merely fat droplets surrounded by a thin shell of protoplasm scarcely more than a membrane Such fat is almost entirely simple lipid in nature and its function is mainly that of serving as an energy store Depot fat decreases in quantity during periods of caloric restriction

About half of the depot fat is deposited in the subcutaneous regions Considerable quantities are located in the mesentery and about the kidneys *In the latter case it serves to provide mechanical support and pro-*



LXIX Arachidonic acid

tection Subcutaneous fat is poorly vascularized and is itself a poor heat conductor It therefore serves as insulation against cold The chemical nature of depot fat varies to a certain extent with the nature of the fat in the diet The subcutaneous fat of a dog fed on linseed oil remains liquid at 0°C as a result of the unusually large quantities of unsaturated acids incorporated therein Fed on mutton fat, the dog's adipose tissue becomes firmer and its melting point higher

Human fat, under normal conditions, is liquid at blood temperature due to a considerable content of oleic acid, linoleic acid, and palmitoleic acid The three together account for about 60 per cent of the fatty acid content of adipose tissue The remaining fatty acid content is almost entirely saturated Palmitic acid is the most common saturated acid, composing about 20 per cent of the total The presence of *arachidonic acid* in small concentration is to be noted Arachidonic acid (formula LXIX) is a highly unsaturated 20 carbon acid, containing four double bonds In rats, at least, its absence in the diet has been shown to cause dermatitis The adrenals are particularly rich in arachidonic acid where it forms 22 per cent of the fatty acid content of their phospholipids

The composition of human depot fat as glycerides rather than as fatty

acids is not yet known, largely due to the difficulty of the chemical separation of these highly similar compounds In the case of ox depot fat, the triglyceride composition has been described as (11)

	molar per cent
Oleopalmitostearin	32
Palmitodiolein	23
Oleodipalmitin	15
Stereodiolein	11
Dipalmitostearin and distearopalmitin	17

MUSCLE

About 40 per cent of the normal human being is muscle, which is thus the largest single tissue component of the body One quarter of the muscle weight is solid matter and of this, four fifths is protein It is the protein content, then, that will first be discussed

Muscle Protein

Actomyosin. This composes 60 per cent of the protein in muscle It is often called simply myosin Actomyosin is the chief, and perhaps the only, protein of the myofibril itself, other muscle proteins occurring in the sarcoplasm Furthermore, actomyosin is *the* contractile substance in muscle, so that studies of muscle contraction have centered upon it Actomyosin is a unique protein, combining within itself the solubility characteristics of a typical globulin and the physical structure of an albuminoid As far as the classical protein classification scheme is concerned, however, solubility properties take precedence and actomyosin is generally included under the globulins

Solutions of actomyosin show marked birefringence of flow and high viscosity, properties characteristic of highly asymmetric molecules X-ray diffraction patterns indicate structures similar to those of keratin, an alpha keratin pattern in normal dried muscle and a beta keratin pattern in stretched muscle Contracted muscle is somewhat analogous to "supercontracted" keratin, except that where hair under supercontraction shrinks to 70 per cent of its normal length, muscle will contract to as little as 30 per cent of its normal length

In the natural state, myofibrils contain actomyosin in the form of long polypeptide chains held together in parallel by subsidiary side chains These are not the cystine disulfide links of keratin, and are undoubtedly more "fluid", allowing a certain degree of sliding It is thought that this is due to the fact that the side chains are polar in nature and highly hydrated In contraction, actomyosin structure "hardens", water is "squeezed out" and the side chains become firmer in their linkage It is difficult to speak

of the molecular weight of actomyosin although perhaps not more difficult than is the case in many other proteins largely because it is impossible to say with precision where in the cell a protein molecule leaves off and another begins Thus the actomyosin micelles within a myofibril have been studied by ultracentrifugation and their size calculated as [illegible] millimicrons in length and 5 millimicrons in thickness This is large enough to hold about twenty actomyosin chains, if the molecular weight is taken to be 1,000 000 However, since these chains are undoubtedly linked in some subsidiary fashion so that the micelle may react as a unit to a stimulus the whole structure may be considered as a macroactomyosin molecule On the other hand, some of the proteins obtained from rabbit muscle show molecular weights of as little as 90 000 (2), although even these fragments retain a high degree of asymmetry Such molecules have been called *tropomyosin* and it is suggested that they represent the individual polypeptide chains of which the micelle is built, larger molecular weights representing chain bundles of various sizes The phenomenon of *rigor mortis* is due to the coagulation of actomyosin after death into a firm insoluble mass Subsequent softening is brought about by autolytic processes

Actomyosin is not a homogeneous protein Straub separated it into two proteins called *actin* and *myosin* Szent Gyorgyi (28) has over many years investigated muscular contraction as a phenomenon related to a reversible complex formation of actin and myosin The complex, now called acto myosin, was previously called myosin The term myosin is thus used in the literature to cover two substances (a) the contractile protein of muscle, and (b) one component of this contractile protein Needless to say a certain confusion has arisen from such practices In this book the component of the actomyosin complex will be referred to as myosin, while the complex itself will be referred to as actomyosin

According to Szent Gyorgyi myosin consists of asymmetric rods 200 to 400 millimicrons long and 2 5 millimicrons wide Myosin is not markedly fibrous The viscosity of its solutions is intermediate between those of solutions of globular proteins and fibrous proteins It is labile and readily denatured Actin is more stable and possesses properties one would expect of a highly fibrous protein Its solutions have extremely high viscosities and show pronounced birefringence of flow The relative proportions of myosin and actin in the actomyosin complex are about eight to three Neither myosin nor actin by itself displays any contractile properties The complex, however, is highly contractile Actin displays one other remarkable property It is capable of being transformed from its highly fibrous state into a normal globular protein of rather small molecular weight Szent Gyorgyi distinguishes between the two forms by naming them *F-actin* and *G actin*, standing for the fibrous and globular forms respec-

tively The molecular weight of G actin is estimated to be 35 000 to 70,000 The conversion of G actin to F actin is probably not due to any unwinding of the globular arrangement, therefore, but to an association of the G actin individuals in a unidimensional manner to form an F actin fiber much as beads may be strung into a necklace The transition between G actin and F actin is reversible and can be repeated many times without damage to the molecule *The conversion of G actin to F actin is catalyzed* by small concentrations of certain metallic ions Monovalent ions will bring about the change at concentrations of 0 1 M, divalent ions at concentrations of 0 005 M Magnesium ions seem to be quite specific in this respect *and in their entire absence polymerization of G actin to F actin* seems not to take place at all If salts are removed by dialysis, F actin will depolymerize to G actin Myosin itself also catalyzes the conversion of G actin to F actin

Myoglobin. A second important protein constituent of muscle tissue is myoglobin the concentration of which in some muscle fibers is as high as 0 8 per cent *It is an iron porphyrin protein that resembles in structure and function the hemoglobin of the blood but is one quarter its molecular weight* Hemoglobin has a molecular weight of about 67,000 and possesses four iron porphyrin groups per molecule (see Chapter 4) Myoglobin has a molecular weight of about 17,500 and contains only one iron porphyrin group per molecule Myoglobin appears in the urine after crushing injury to the muscles

It should not be assumed from the foregoing that myoglobin is nothing but a ' quarter hemoglobin ' molecule While it is true that hemoglobin may readily be split into semi molecules in the presence of solutions of substances such as urea and guanidine and that each semi molecule contains two iron porphyrin groups, remains functional and can reform into the original hemoglobin on removal of the urea or guanidine by dialysis, a further reversible splitting of the semi hemoglobin molecules into functional quarter molecules has not yet been successfully achieved Myoglobin, while closely resembling what one would expect a quarter hemoglobin to

The

avail

in having a some

n partial pressure,

s 60 per cent satu

l to the contracting

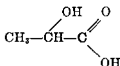
in, which absorbs

up in turn to the

respiratory enzyme systems of the cells which have a still greater affinity for oxygen

Muscle Carbohydrate

Glycogen. Although the liver is generally considered the organ in which glycogen is chiefly stored and although it is truly the organ in which that carbohydrate is present in the highest concentration (as high as 6 per cent of the organ), the *total* glycogen content of resting muscle is greater than that of liver, since the muscles of the body have a total weight some fifteen times that of the liver The glycogen concentration of resting muscle is 0 5 to 1 0 per cent, but decreases during muscular activity and may approach the vanishing point when the activity is particularly intense and prolonged In the complete absence of glycogen, normal muscular activity can not continue Together, glycogen and myoglobin represent an autonomous energy system (one supplying carbon and hydrogen and the other oxygen) for the private use of this extremely active and responsive tissue



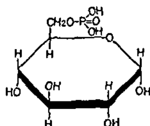
LXX Lactic acid

Carbohydrate metabolic products. The utilization of glycogen by muscle takes place via a series of phosphorylative and oxidative steps which, in the presence of adequate oxygen, is continued to the final conversion of the carbohydrate to carbon dioxide and water During vigorous muscular exercise, the accelerated utilization of glycogen is more than the available supply of oxygen can take care of Energy is then obtained by anaerobic glycolysis (see Chapter 12) in which case the end product is *lactic acid* (formula LXX) The accumulating lactic acid must eventually be oxidized or reconverted to glycogen if muscular activity is to continue Resting muscle contains about 0 02 per cent of lactic acid, while during exercise the concentration rises rapidly to about 0 25 per cent Concentrations as high as 0 4 per cent have been detected in muscle artifically put into rigor by heat, injury, or chloroform, and allowed to remain so to death of the tissue, or by complete deprivation of oxygen to the point of tissue death (31)

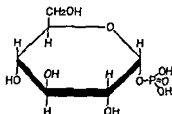
Small quantities of intermediates in carbohydrate metabolism, other than lactic acid, have also been detected in muscle The chief of these is *hexosephosphate*

Actually this substance is not a chemical individual Conway and

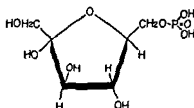
Hingerty (5) in analyzing rat muscle found it to contain about 10 millimoles of hexosephosphate per kilogram (0 26 per cent) and that this consisted of glucose 6 phosphate, glucose-1-phosphate, and fructose-6 phosphate in the proportions of approximately 6·3:1 (formula LXXI) At the time of the first discovery of hexosephosphate, when it was thought to be a chemical individual, it received the name *Embden ester*, by which it is still sometimes known in medical literature Glucose 6 phosphate, glucose 1-phosphate, and fructose 6 phosphate are known individually as *Robison*



GLUCOSE-6-PHOSPHATE
(ROBISON ESTER)



GLUCOSE-1-PHOSPHATE
(CORI ESTER)



FRUCTOSE-6-PHOSPHATE
(NEUBERG ESTER)
LXXI

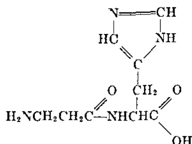
ester, *Cori ester*, and *Neuberg ester* In addition to the hexosephosphate, there were found present in muscle about 3 5 millimoles of triose phosphates per kilogram (0 06 per cent)

Non-Protein Nitrogen Constituents

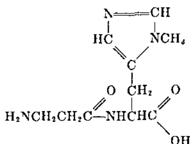
Two water soluble phosphorus containing substances, *phosphocreatine* (also called creatine phosphate or phosphagen) and *adenosine triphosphate* (referred to for convenience's sake as ATP), occur in appreciable concentration in muscle Conway and Hingerty found 21 4 millimoles of phosphocreatine per kilogram of rat muscle (0 5 per cent) and 19 5 millimoles of ATP phosphorus or 6 5 millimoles of ATP itself per kilogram of rat muscle (0 4 per cent) Both play a vital role in the chemistry of muscular

contraction (see p 148) Significant differences in ATP and phosphocreatine content of various types of muscle have been found Mommaerts (17) states that cardiac muscle contains only one third the concentration of ATP that skeletal muscle contains and only one tenth the concentration of phosphocreatine

Two dipeptides of unknown function occur in muscle *Carnosine* (formula LXXII) is a dipeptide of histidine and beta alanine The latter amino acid is an unusual example of a naturally occurring amino acid with no



LXXII Carnosine



LXXIII Anserine

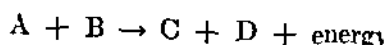
amino group in the alpha position It is further interesting to note that beta alanine also occurs in the food factor, pantothenic acid, one of the B vitamins (see Chapter 19) *Anserine* (formula LXXIII) is a methyl carnosine Their quantitative occurrence in rat muscle in millimoles per kilogram of tissue is 1 4 and 20 3, which in percentage is 0 03 and 0 48 for carnosine and anserine, respectively

The High-Energy Phosphate Bond

Before describing the functions of adenosine triphosphate and phosphocreatine, it is necessary to discuss the nature and functioning of high energy phosphate bonds

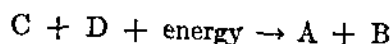
All chemical reactions involve the liberation or absorption of energy

A general chemical reaction could thus be written as follows



$$A + B \rightarrow C + D + \text{energy}$$

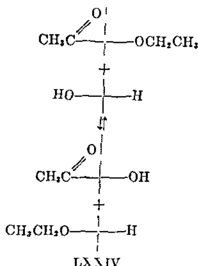
The reaction is ideally reversible



$$C + D + \text{energy} \rightarrow A + B$$

Energy is neither created nor destroyed in the process If a mol of A plus a mol of B form a mol of C and a mol of D plus x calories, then to convert a mol of C and one of D back into the original quantity of A and B requires an *input* of x calories

But if energy is neither created nor destroyed, where does it come from?

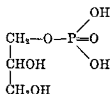


LXXIV

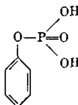
hat happens to it when it is put into C and D to make A and B? This will take a little time to answer It is necessary to remember that in any chemical reaction certain bonds between atoms are broken and new ones are formed Thus in the hydrolysis of ethyl acetate by water, two bonds are broken—*the ethyl to acetate bond of the ester and the hydrogen to* hydroxyl bond of the water (formula LXXIV) Two new bonds an ethyl to hydroxyl bond and a hydrogen to acetate bond are formed Various bonds may be considered as possessing different energy contents Generally speaking this energy content will vary inversely with the ease with which a particular bond is formed

Thus, oxygen and hydrogen have a strong tendency to form a chemical bond In fact, oxygen and hydrogen have a stronger tendency to form a bond with one another than either has to form one with another atom of itself Hydrogen and oxygen will therefore combine with one another to

form water with the liberation of energy, the energy arising from the increased stability (hence, smaller energy content) of the two O—H bonds as compared with the O—O bond plus the H—H bond Conversely, the process whereby the O—H bonds are broken and the O—O and H—H bonds reconstituted, that is, the decomposition of water to hydrogen and oxygen is one which requires an equivalent input of energy The reverse situation is the case where the nitrogen oxides are concerned Here the O—N bond is less stable than the O—O and N—N bonds and energy must be supplied to form nitric oxide (NO) and energy is liberated in its decomposition to oxygen and nitrogen The whole may be viewed thus the energy required to build a molecule out of its constituent atoms is frozen in the molecule and can be termed "chemical energy" In any process which changes one group of atoms to another group of atoms, the difference in chemical



Glyceryl phosphate



Phenyl phosphate

LXXV Low-energy phosphate bonds

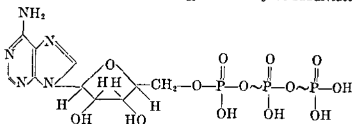
energy of the two groups is accounted for by its equivalent in other forms of energy which is liberated by the reaction or which must be supplied

The chemical energy of a given bond, say that between carbon and oxygen varies somewhat with the structure of the molecule as a whole The variation of energy of a given type of bond is usually not great among substances of biochemical interest One important exception to this generalization does exist, however, and that involves the bonds whereby the phosphorus of phosphoric acid is attached to the oxygen or nitrogen of an organic substance

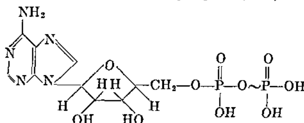
Phosphate bonds fall into two large groups differing widely in their energy content One group, known as "low energy phosphate bonds" are more stable, easier to form, harder to hydrolyze and for those very reasons have a lower energy content This group of compounds includes the *phosphate esters of alcohols and phenols* Typical examples of compounds containing low-energy phosphate bonds are glyceryl phosphate and phenyl phosphate (formula LXXV) The various glucose and fructose phosphates which are of importance in carbohydrate metabolism (see Chapter 12) also contain low-energy bonds

A second group, the "high-energy phosphate bonds" is less stable,

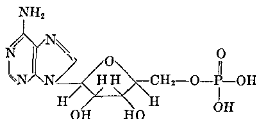
harder to form, and easier to hydrolyze, and for those very reasons has a higher energy content This group of compounds includes *acid anhydrides of phosphoric acid and those compounds containing a phosphorus nitrogen bond* The group containing high energy bonds may be subdivided further



LXXVI Adenosine triphosphate (ATP)



LXXVII Adenosine diphosphate (ADP)



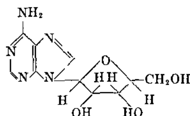
LXXVIII Adenylic acid (AMP)

and in view of the great importance of these compounds in metabolism, they will be taken up in some detail

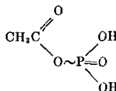
1 Acid anhydride formation between two molecules of phosphoric acid to form a *pyrophosphoric link* Compounds containing such links may be typified by ATP and *adenosine diphosphate* (ADP) (formulas LXXVI and LXXVII) It is conventional in writing these and other such formulas to use a wavy line (~) to indicate a high energy phosphate bond Note that ATP has two such pyrophosphate links In breaking such a link, as in the hydrolysis of ATP to ADP or ADP to *adenylic acid* (formula LXXVIII) (also called adenosine monophosphate, or AMP), comparatively large

quantities of energy are released The hydrolysis of adenylic acid, which has only a low-energy phosphate bond remaining, to *adenosine* (formula LXXIX), releases only about one quarter as much energy per bond High energy pyrophosphate bonds are also found in various coenzymes (see Chapter 5)

2 Acid anhydride formation between a molecule of phosphoric acid and one of a carboxylic acid Such compounds are acyl phosphates and an example is *acetyl phosphate* (formula LXXX) which some consider to be related to the fundamental two carbon building block of the body through which fat metabolism carbohydrate metabolism and protein metabolism are interlinked (see Chapter 12)



LXXIX Adenosine



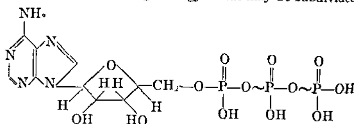
LXXX Acetyl phosphate

3 Acid anhydride formation between a molecule of phosphoric acid and an enol form of a keto acid (formula LXXXI) An example is *phosphoenolpyruvic acid* (formula LXXXII), which is a step in the glycolytic breakdown of glycogen

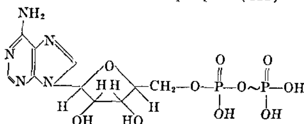
4 A compound containing a direct N—P bond The most important such compound in the vertebrate body is phosphocreatine (formula LXXXIIIa), already mentioned as a muscle component Note that the phosphate group is attached to the nitrogen of a guanidino group Arginine contains such a guanidino group and, in fact, in invertebrate muscle, phosphocreatine does not exist and *phosphoarginine* (formula LXXXIIIb), or arginine phosphate as it is sometimes called, is found instead

The function of the high energy phosphate bond in animal metabolism is to act as a store of readily available energy If ATP, for instance, is hydrolyzed to ADP, energy is released which can be utilized to force a

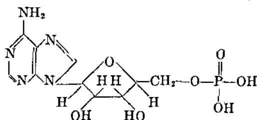
harder to form, and easier to hydrolyze, and for those very reasons has a higher *energy content* This group of compounds includes *acid anhydrides of phosphoric acid and those compounds containing a phosphorus nitrogen bond* The group containing high energy bonds may be subdivided further



LXXVI Adenosine triphosphate (ATP)



LXXVII Adenosine diphosphate (ADP)



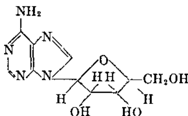
LXXVIII Adenylic acid (AMP)

and in view of the great importance of these compounds in metabolism, they will be taken up in some detail

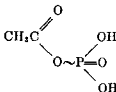
1 Acid anhydride formation between two molecules of phosphoric acid to form a *pyrophosphoric link* Compounds containing such links may be typified by ATP and *adenosine diphosphate* (ADP) (formulas LXXVI and LXXVII) It is conventional in writing these and other such formulas to use a wavy line (~) to indicate a high energy phosphate bond Note that ATP has two such pyrophosphate links In breaking such a link, as in the hydrolysis of ATP to ADP or ADP to *adenylic acid* (formula LXXVIII) (also called adenosine monophosphate, or AMP), comparatively large

quantities of energy are released The hydrolysis of adenylic acid, which has only a low energy phosphate bond remaining, to *adenosine* (formula LXXIX), releases only about one quarter as much energy per bond High energy pyrophosphate bonds are also found in various coenzymes (see Chapter 5)

2 Acid anhydride formation between a molecule of phosphoric acid and one of a carboxylic acid Such compounds are acyl phosphates and an example is *acetyl phosphate* (formula LXXX) which some consider to be related to the fundamental two carbon building block of the body through which fat metabolism, carbohydrate metabolism, and protein metabolism are interlinked (see Chapter 12)



LXXIX Adenosine



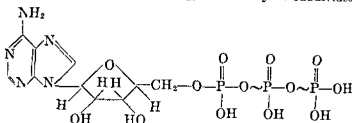
LXXX Acetyl phosphate

3 Acid anhydride formation between a molecule of phosphoric acid and an enol form of a keto acid (formula LXXXI) An example is *phosphoenolpyruvic acid* (formula LXXXII), which is a step in the glycolytic breakdown of glycogen

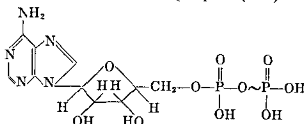
4 A compound containing a direct N—P bond The most important such compound in the vertebrate body is phosphocreatine (formula LXXXIIIa), already mentioned as a muscle component Note that the phosphate group is attached to the nitrogen of a guanidino group Arginine contains *such a guanidino group and, in fact, in invertebrate muscle,* phosphocreatine does not exist and *phosphoarginine* (formula LXXXIIIb), or arginine phosphate as it is sometimes called, is found instead

The function of the high-energy phosphate bond in animal metabolism is to act as a store of readily available energy If ATP, for instance, is hydrolyzed to ADP, energy is released which can be utilized to force a

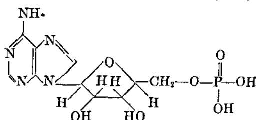
harder to form, and easier to hydrolyze, and for those very reasons has a higher energy content This group of compounds includes *acid anhydrides of phosphoric acid and those compounds containing a phosphorus nitrogen bond* The group containing high-energy bonds may be subdivided further



LXXVI Adenosine triphosphate (ATP)



LXXVII Adenosine diphosphate (ADP)



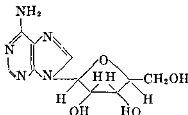
LXXVIII Adenylic acid (AMP)

and in view of the great importance of these compounds in metabolism, they will be taken up in some detail

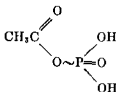
1 Acid anhydride formation between two molecules of phosphoric acid to form a *pyrophosphoric link* Compounds containing such links may be typified by ATP and *adenosine diphosphate* (ADP) (formulas LXXVI and LXXVII) It is conventional in writing these and other such formulas to use a wavy line (~) to indicate a high energy phosphate bond Note that ATP has two such pyrophosphate links In breaking such a link, as in the hydrolysis of ATP to ADP or ADP to *adenylic acid* (formula LXXVIII) (also called adenosine monophosphate, or AMP), comparatively large

quantities of energy are released The hydrolysis of adenylic acid, which has only a low energy phosphate bond remaining, to *adenosine* (formula LXXIX), releases only about one quarter as much energy per bond High energy pyrophosphate bonds are also found in various coenzymes (see Chapter 5)

2 Acid anhydride formation between a molecule of phosphoric acid and one of a carboxylic acid Such compounds are acyl phosphates and an example is *acetyl phosphate* (formula LXXX) which some consider to be related to the fundamental two carbon building block of the body through which fat metabolism carbohydrate metabolism, and protein metabolism are interlinked (see Chapter 12)



LXXIX Adenosine



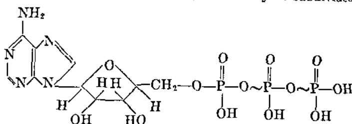
LXXX Acetyl phosphate

3 Acid anhydride formation between a molecule of phosphoric acid and an enol form of a keto acid (formula LXXXI) An example is *phosphoenolpyruvic acid* (formula LXXXII), which is a step in the glycolytic breakdown of glycogen

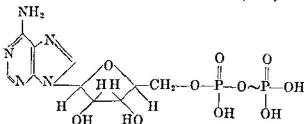
4 A compound containing a direct N—P bond The most important such compound in the vertebrate body is phosphocreatine (formula LXXXIIIa), already mentioned as a muscle component Note that the phosphate group is attached to the nitrogen of a guanidino group Arginine contains *such a guanidino group and, in fact, in invertebrate muscle, phosphocreatine does not exist and phosphoarginine* (formula LXXXIIIb), or arginine phosphate as it is sometimes called, is found instead

The function of the high-energy phosphate bond in animal metabolism is to act as a store of readily available energy If ATP, for instance, is hydrolyzed to ADP, energy is released which can be utilized to force a

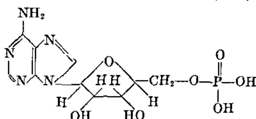
harder to form, and easier to hydrolyze, and for those very reasons has a higher energy content This group of compounds includes *acid anhydrides of phosphoric acid and those compounds containing a phosphorus nitrogen bond* The group containing high energy bonds may be subdivided further



LXXVI Adenosine triphosphate (ATP)



LXXVII Adenosine diphosphate (ADP)



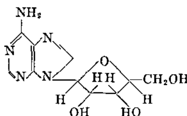
LXXVIII Adenylic acid (AMP)

and in view of the great importance of these compounds in metabolism, they will be taken up in some detail

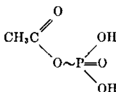
1 Acid anhydride formation between two molecules of phosphoric acid to form a *pyrophosphoric link* Compounds containing such links may be typified by ATP and *adenosine diphosphate* (ADP) (formulas LXXVI and LXXVII) It is conventional in writing these and other such formulas to use a wavy line (~) to indicate a high energy phosphate bond Note that ATP has two such pyrophosphate links In breaking such a link, as in the hydrolysis of ATP to ADP or ADP to *adenylic acid* (formula LXXVIII) (*also called adenosine* monophosphate, or AMP), comparatively large

quantities of energy are released The hydrolysis of adenylic acid, which has only a low energy phosphate bond remaining, to *adenosine* (formula LXXIX), releases only about one quarter as much energy per bond High energy pyrophosphate bonds are also found in various coenzymes (see Chapter 5)

2 Acid anhydride formation between a molecule of phosphoric acid and one of a carboxylic acid Such compounds are acyl phosphates and an example is *acetyl phosphate* (formula LXXX) which some consider to be related to the fundamental two carbon building block of the body through which fat metabolism, carbohydrate metabolism, and protein metabolism are interlinked (see Chapter 12)



LXXIX Adenosine



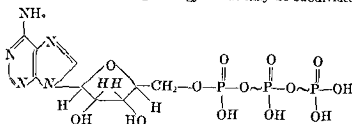
LXXX Acetyl phosphate

3 Acid anhydride formation between a molecule of phosphoric acid and an enol form of a keto acid (formula LXXXI) An example is *phosphoenolpyruvic acid* (formula LXXXII), which is a step in the glycolytic breakdown of glycogen

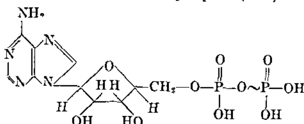
4 A compound containing a direct N—P bond The most important such compound in the vertebrate body is phosphocreatine (formula LXXXIIIa), already mentioned as a muscle component Note that the phosphate group is attached to the nitrogen of a guanidino group Arginine contains such a guanidino group and, in fact, in invertebrate muscle, *phosphocreatine does not exist and phosphoarginine (formula LXXXIIIb),* or arginine phosphate as it is sometimes called, is found instead

The function of the high-energy phosphate bond in animal metabolism is to act as a store of readily available energy If ATP, for instance, is hydrolyzed to ADP, energy is released which can be utilized to force a

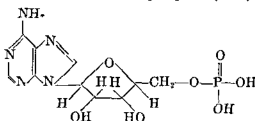
harder to form, and easier to hydrolyze, and for those very reasons has a higher energy content This group of compounds includes *acid anhydrides of phosphoric acid and those compounds containing a phosphorus nitrogen bond* The group containing high energy bonds may be subdivided further



LXXVI Adenosine triphosphate (ATP)



LXXVII Adenosine diphosphate (ADP)



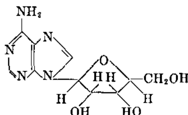
LXXVIII Adenylic acid (AMP)

and in view of the great importance of these compounds in metabolism, they will be taken up in some detail

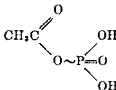
1 Acid anhydride formation between two molecules of phosphoric acid to form a *pyrophosphoric link* *Compounds containing such links may be* typified by ATP and *adenosine diphosphate* (ADP) (formulas LXXVI and LXXVII) It is conventional in writing these and other such formulas to use a wavy line (~) to indicate a high energy phosphate bond Note that ATP has two such pyrophosphate links In breaking such a link, as in the hydrolysis of ATP to ADP or ADP to *adenylic acid* (formula LXXVIII) (also called adenosine monophosphate, or AMP), comparatively large

quantities of energy are released The hydrolysis of adenylic acid, which has only a low-energy phosphate bond remaining to *adenosine* (formula LXXIX), releases only about one quarter as much energy per bond High energy pyrophosphate bonds are also found in various coenzymes (see Chapter 5)

2 Acid anhydride formation between a molecule of phosphoric acid and one of a carboxylic acid Such compounds are acyl phosphates and an example is *acetyl phosphate* (formula LXXX) which some consider to be related to the fundamental two carbon building block of the body through which fat metabolism carbohydrate metabolism and protein metabolism are interlinked (see Chapter 12)



LXXIX Adenosine



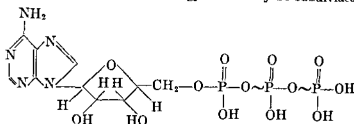
LXXX Acetyl phosphate

3 Acid anhydride formation between a molecule of phosphoric acid and an enol form of a keto acid (formula LXXXI) An example is *phosphoenolpyruvic acid* (formula LXXXII), which is a step in the glycolytic breakdown of glycogen

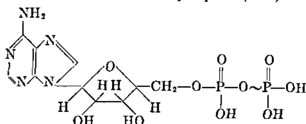
4 A compound containing a direct N—P bond The most important such compound in the vertebrate body is phosphocreatine (formula LXXXIIIa), already mentioned as a muscle component Note that the phosphate group is attached to the nitrogen of a guanidino group Arginine contains such a guanidino group and, in fact, in invertebrate muscle, phosphocreatine does not exist and *phosphoarginine* (formula LXXXIIIb) or arginine phosphate as it is sometimes called, is found instead

The function of the high-energy phosphate bond in animal metabolism is to act as a store of readily available energy If ATP, for instance, is hydrolyzed to ADP, energy is released which can be utilized to force a

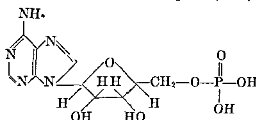
harder to form, and easier to hydrolyze, and for those very reasons has a higher energy content This group of compounds includes *acid anhydrides of phosphoric acid and those compounds containing a phosphorus nitrogen bond* The group containing high-energy bonds may be subdivided further



LXXVI Adenosine triphosphate (ATP)



LXXVII Adenosine diphosphate (ADP)



LXXVIII Adenylic acid (AMP)

and in view of the great importance of these compounds in metabolism, they will be taken up in some detail

1 Acid anhydride formation between two molecules of phosphoric acid to form a *pyrophosphoric link* Compounds containing such links may be typified by ATP and *adenosine diphosphate* (ADP) (formulas LXXVI and LXXVII) It is conventional in writing these and other such formulas to use a wavy line (~) to indicate a high-energy phosphate bond Note that ATP has two such pyrophosphate links In breaking such a link, as in the hydrolysis of ATP to ADP or ADP to *adenylic acid* (formula LXXVIII) (also called adenosine monophosphate, or AMP), comparatively large

The reverse of such a reaction (that is the formation of ATP from ADP by hydrolysis of glucose 6 phosphate to glucose) can not take place unless

GLUCOSE + ATP

GLUCOSE 6 PHOSPHATE + ADP

LXXXIV

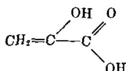
1 3 Diphosphoglyceraldehyde —Oxidation − 2H→ 1 3 Diphosphoglyceric acid

LXXXV

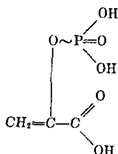
the difference in energy, 7 to 10 Kcal is supplied since in thermodynamics even more than in economics one can get nothing for nothing In the body, energy input of such magnitude can not be supplied except by the utilization of another high-energy bond

Some way must exist, however, of forming high-energy phosphate bonds

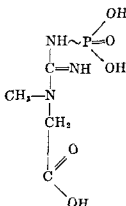
second reaction, requiring energy uptake, to completion An example of such a two reaction system is the formation of glucose 6 phosphate from glucose in the presence of the appropriate enzyme when ATP is added



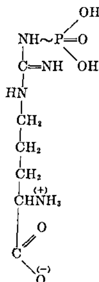
LXXXI
Pyruvic acid (enol form)



LXXXII.
Phosphoenolpyruvic acid



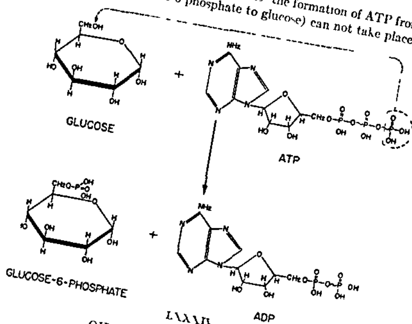
LXXXIIIa
Phosphocreatine



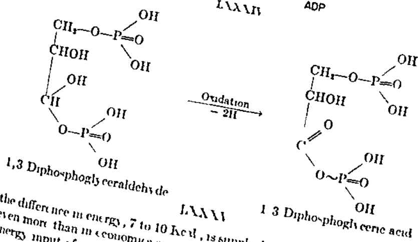
LXXXIIIb.
Phosphoarginine

(formula LXXXIV) The reaction does not take place *in the absence of* ATP Note that the phosphate bond formed on the glucose molecule is low-energy Since one mol of high-energy phosphate bonds yields upon hydrolysis 10 to 12 Kcal *while one mol of low-energy phosphate bonds* yields only
formed at

The reverse of such a reaction (that is the formation of ATP from ADP by hydrolysis of glucose 6 phosphate to glucose) can not take place unless



LXXXIV

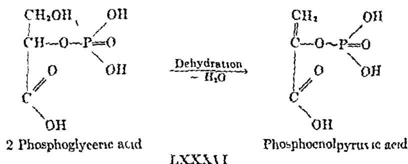


LXXXV

the difference in energy, 7 to 10 Kcal, is supplied since in thermodynamics even more than in economics one can get nothing for nothing In the body, energy input of such magnitude can not be supplied except by the utilization of another high-energy bond

Some way must exist however, of forming high-energy phosphate bonds

without the utilization of such a bond, as otherwise the body's limited supply of such bonds would be quickly consumed with fatal results The formation of such bonds takes place for example, during the metabolism of glycogen where the energy liberated in certain reactions is "stored" in the form of such bonds That is, a compound containing a low-energy bond is converted by oxidation or dehydration into one of a structure required for high-energy bonds An example of two such reactions which take place in the glycolytic breakdown of glycogen to lactic acid are (a) the oxidation of 1 3 diphosphoglyceraldehyde (low-energy) to 1 3-diphosphoglyceric acid (high-energy) (formula LXXXV), and (b) the dehydration of 2 phosphoglyceric acid (low-energy) to phosphoenolpyruvic acid (high energy) (formula LXXXVI) Both 1 3-diphosphoglyceric acid and phosphoenolpyruvic acid are capable of transferring enough energy on hydrolysis to form ATP from ADP



LXXXVI

Muscular Contraction

Chemical aspects. It has already been stated that two substances containing high-energy phosphate bonds, ATP and phosphocreatine, are found in muscle The energy derived from the hydrolysis of these com

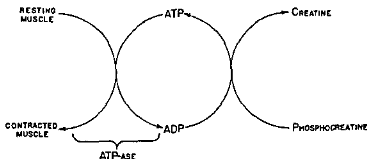
the enzyme *adenosinetriphosphatase*, or ATP ase

The ATP ase of muscle is closely associated with actomyosin, so closely associated that separation of the two by present methods of protein fractionation has not yet been satisfactorily accomplished Many consider ATP ase to be *identical* with actomyosin If this is true, ATP ase is the most abundant enzyme in the body, since about one twenty fifth of the body weight is actomyosin It is this abundance of actomyosin which makes its identity with ATP ase most suspect, since all other enzymes in the human body are present in much lower concentrations

Only *after* at least some of the ATP present in muscle is hydrolyzed to

ADP, does phosphocreatine take part in the process It reacts with ADP, forming ATP once again by phosphate transfer, being itself converted to creatine This reaction is reversible, since the high-energy bonds in the two compounds are about equivalent in energy content With ATP disappearing in the process of muscular contraction however, the reaction is forced in the direction indicated The relationship of the two reactions may be shown by means of Baldwin cycles (see page 243) It will be noted that a molecule of ATP is alternately hydrolyzed and re formed so that a great deal of energy may be transferred through a relatively small amount of ATP which acts as an energy shuttle" (formula LXXXVII)

Why the necessity for this two step hydrolysis process? In the first place phosphocreatine acts as a high energy phosphate bond reserve One hundred grams of rat muscle, for instance, contains 20 milligrams of high-energy



LXXXVII

phosphate derivable from the conversion of ATP to ADP and 75 milligrams nearly four times as much, of high-energy phosphate derivable from the conversion of phosphocreatine to creatine It might be supposed that since the phosphate bonds of ATP and phosphocreatine are virtually equivalent, the entire 95 milligrams of phosphate might as well be all one or all the other—and the process thus simplified Actually, there is an important difference between the two compounds The ATP ase in muscle catalyzes the hydrolysis of ATP only, it has no effect upon phosphocreatine Thus, if all the high energy phosphate were in the form of phosphocreatine then, utilizing actual muscle biochemical mechanisms, none of its energy could be made available for contraction On the other hand, if the high energy phosphate were all in the form of ATP, the effect of the ATP ase would be to explode that energy too rapidly The initial impulse which sets off the ATP is limited by the fact that only a small quantity of ATP is present Action, thereafter, is continued in a more controlled fashion by allowing the phosphocreatine ADP reaction to act as a bottleneck

The total supply of ATP and phosphocreatine together is insufficient to

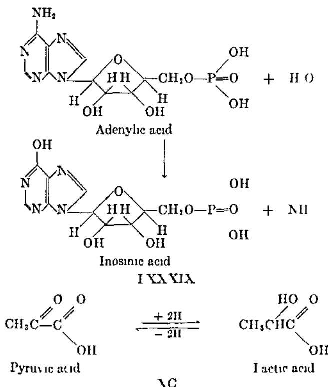
ADP + ADP

[myokinase]

ATP + Adenylic acid (AMP)

LXXXVIII

support muscular contraction for more than a very limited time However ATP can be resynthesized through the glycolytic breakdown of muscle glycogen If a muscle is poisoned with iodoacetate (which inhibits glycolysis) ATP resynthesis is prevented and on repeated stimulation of the muscle its ATP will be entirely converted to ADP ADP as a last gasp may be reconverted *in part* to ATP through the action of myokinase an enzyme



found in muscle which builds up a molecule of ADP to ATP at the expense of hydrolyzing a second molecule to adenylic acid (AMP) (formula LXXXVIII) This is possible since ADP still has one high-energy phosphate bond which can be utilized for the transfer However this is only the most temporary of solutions since the AMP formed is deaminated to inosinic acid and can then no longer re-enter the phosphate cycle (formula LXXXIX) At each step of resynthesis of ATP by this method therefore half the remaining available phosphorus supply is lost and very quickly the muscle goes into final rigor

In a muscle in normal contraction, glycogen is broken down and ATP

is resynthesized through the formation of high-energy phosphate bonds during glycolysis Phosphoenolpyruvic acid formed during glycolysis can donate its phosphate group to ADP, resynthesizing ATP and itself being converted to *pyruvic acid* *Pyruvic acid can be eventually oxidized to* carbon dioxide and water if there is adequate molecular oxygen Where, as in a muscle during strenuous exercise, the oxygen supply is insufficient to oxidize the pyruvic acid as quickly as it is formed from glycogen break down, pyruvic acid is reduced to lactic acid (formula XC)

What, then, is the *function of the phosphocreatine since the phosphoenol* pyruvic acid formed from glycogen breakdown can substitute for it? Actually, glycolysis is a comparatively slow starting reaction and the need for muscular activity may be very urgent indeed The phosphocreatine acts as an energy reserve which is ready at the moment and which can bridge *the gap between the time of stimulus and the time when glycolysis* is proceeding with sufficient rapidity When muscular activity is over, there is a short period *of anaerobic recovery* during which glycogen continues to break down and lactic acid to accumulate—but the high energy phosphate bonds are expended, not in resynthesizing ATP which for the moment is no longer being broken down, but in resynthesizing the phosphocreatine from creatine and thus restoring the original high-energy bond situation

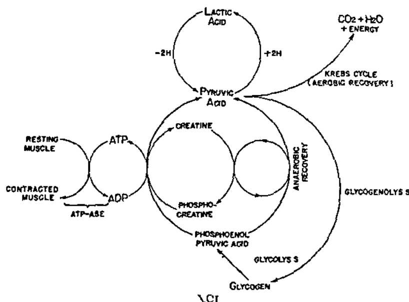
There remains the resynthesis of glycogen While the energy available within the muscle from glycogen breakdown is about five times the sum of that available from muscle ATP and phosphocreatine, it too is insufficient in extreme cases A strenuously exercised muscle is eventually depleted *of glycogen, crammed with lactic acid, and weary to exhaustion* It has been living on its capital, and the capital is gone In order for the muscle to recover, the lactic acid must be reconverted to glycogen This takes energy, and the energy is gained by the complete oxidation, aerobically, of lactic acid to carbon dioxide and water Until this step, the various chemical changes in contracting muscle have not required molecular oxygen, glycolysis being an *anaerobic* process The muscle after prolonged stimulation has thus incurred what *is termed an oxygen debt*, and [illegible] *aerobic recovery* [illegible]

[illegible] on by first dehydrogenating it to [illegible]

[illegible] cycle will be *found in* Chapter 12 This aerobic [illegible] produces considerably more energy per three carbon unit than did the initial breakdown of glycogen to lactic acid *The complete oxidation of* one fifth of the lactic acid in the muscle will supply sufficient energy to reconvert the other four fifths of the lactic acid to glycogen From this

final picture of the chemical changes involved in muscular contraction it will be seen that ultimately the energy is derived from the aerobic oxidation of glycogen to carbon dioxide and water (formula XCI) In muscles performing moderate activity aerobic oxidation keeps up with the energy demands

Physical aspects Granted that the hydrolysis of ATP liberates the energy necessary for muscular contraction what physical changes take place in the muscle as a result? Szent Gyorgyi (28) found that actomyosin threads would contract in the presence of K^+ but would do so much more



XCI

sharply and intensely in the presence of both K^+ and ATP Even a solution of actomyosin in the presence of K^+ and ATP will coagulate into a small hard plug showing that the protein molecule itself is the contractile element rather than any structural element larger than the molecule Szent Gyorgyi postulates the actomyosin complex to be built up of a thread like myosin backbone along which is strung a molecule of F actin which in turn is a partially joined conglomerate of G actin globules For simplicity's sake let us assume this to be a linear complex

The action of K^+ and ATP is such as to encourage the dehydration of both the F actin and myosin moieties of the complex In so doing the length of each moiety decreases and if the dehydration of myosin is considered to be a little more extensive than that of F actin then in order for

the two partners to remain joined all along their lengths curvature must take place This phenomenon is analogous to the curvature with temperature change of a bimetallic strip composed of two metals with different coefficients of linear expansion Where dehydration results in a difference in length of 3 per cent between myosin and F actin the whole complex would be forced to bend in a circle assuming the original ratio of length of the molecule to the width to be 100 The final circle would be shorter and thicker than the original line The same effect can be shown to take place if the actomyosin molecule is a helix rather than a straight line Differential dehydration would result in a shorter and thicker helix

Undoubtedly this is a vast oversimplification of the case So far neither this hypothesis nor any other has met with general approval on the part of muscle biochemists Nevertheless the Szent Gyorgyi hypothesis is worth presenting if only because it demonstrates one possible mechanism whereby a relatively small chemical change can result in a relatively large physical one of the type required to explain muscular contraction

NERVE TISSUE

Proteins

The fact that nerve tissue originates from the ectoderm renders somewhat less surprising the fact that one of the important proteins of nerve issue is an albuminoid *neurokeratin* closely related to keratin Chemically t differs from ordinary keratins chiefly in that the arginine content is onsiderably smaller In the brain and in the nervous system generally ieurokeratin fulfills a connective function being the chief constituent of he fibrils (neuroglia) which are the supporting tissues of the brain

Other protein components of neural tissue include several globulins wo of which coagulate at the unusually low temperature of 47°C In ddition nerve cytoplasm is unusually rich in *pentosenucleoprotein* (see hapter 7) which probably plays some role in nerve conduction since it s reported to disappear in part on nerve stimulation (19) Monné (19) peculates that the high nucleoprotein content of nerve cytoplasm is neces ary for the ready formation upon demand of a vast variety of highly pecific proteins (perhaps the relatively unstable globulins already referred o) by means of which elusive processes as memory conception and imagi ation are made possible Each new idea or experience might be stored n a particular protein molecule the brain as a whole functioning in a ianner similar to the cybernetic machines of modern mathematical tech ology Instinct would be interpreted in such a scheme as the inheritance f specific nucleoproteins adapted to prepare certain pre-established pro- ins from generation to generation (The general role of nucleoprotein in he biosynthesis of proteins is discussed in Chapter 7)

Nerve Lipids

Nerve tissue is remarkable for the wealth and variety of lipid material found within it. Of the solid matter of brain about 40 per cent is protein and more than 50 per cent lipid, practically all of the latter being compound or associated lipids. Of the lipid content of the cerebrum, for instance, 52 per cent is phospholipid in nature, 19 per cent is cholesterol and 13 per cent is galactolipid. The remainder is composed largely of poorly characterized inositides and sulfur containing lipids.

The myelin sheaths are particularly rich in lipid. Here their function is probably one of insulation. The nerve impulse is accompanied by electrical phenomena and any device which will prevent loss of electrical potential by leakage will enable a smaller current to be effective and hence permit a thinner nerve fiber. Vertebrate axons, for instance, are only a few microns in diameter so that a two inch length will weigh one hundredth of a milligram. A squid, on the other hand, the nerves of which are not surrounded by a lipid filled myelin sheath, must prevent potential loss and maintain efficiency by lowering the resistance of the neural paths. This is done by thickening the axon to, in some cases, nearly a millimeter. A two-inch length of such an axon would weigh 10 to 20 milligrams. For this reason much chemical and physiological research concerns itself with the nervous system of the squid, a creature which is not at all closely related to man.

Monné (19) cites reasons for believing that the compound lipids of the cell have as their chief function that of insulating enzyme systems from one another, and until necessary, individual enzymes from their substrates (see Chapter 5). Thus, the myriad reactions within a cell would not occur at random but would be moderated and organized by the presence or removal of the compound lipid barriers. The importance of compound lipids to cellular metabolism is pointed up by the fact that at least one of the active principles of the venom of bees, scorpions, and some snakes, such as the cobra and rattlesnake, is a lecithinase capable of hydrolyzing one of the fatty acids away from lecithin or cephalin leaving behind *lysolecithin* and *lysocephalin*, respectively. The painful, and sometimes fatal, results of such a change in the lecithin and cephalin structures are well known.

Nerve Conduction

Potassium ion. *In the living nerve cell, potassium accumulates within* the cell in great excess, the ratio of concentrations of cellular to extracellular potassium in the giant axon of the squid has been found to be as high as 29 (3, 30). Experiments with radioactive potassium and sodium showed the cell membrane of erythrocytes to be equally permeable to both, although only the former accumulated within the cell. No entirely satis-

factory theory exists to explain the accumulation of potassium against a concentration gradient but the current concept is that potassium forms a protein complex within the cell (27) thus becoming part of a system to which the cell membrane is impermeable, shifting the equilibrium in favor of continued influx of potassium Such bound *potassium is in equilibrium* with ionic potassium as isotope exchange experiments show For a fuller discussion of the subject the student is referred to a review by Sheppard (*24*)

The difference in concentration between potassium within and without the cell causes a potential difference to exist across the membrane since the system, nerve cell/surrounding medium, possesses the characteristics of what is known in electrochemistry as a "concentration cell" A membrane across which such a potential difference *exists is known as a polarized membrane*

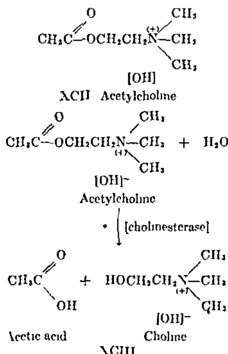
The potential difference across the nerve membrane has been found to be between *75 and 95 millivolts* The maintenance of such a difference, of course requires energy which is derived from the metabolism of glucose within the cell A dead nerve cell is unable to maintain the potential difference and the potassium concentration equalizes across the membrane which is then said to be *depolarized* If a section of a nerve fiber is injured, that part is depolarized and a potential is set up between it and the remaining uninjured portions A nerve impulse is accompanied by a naturally occurring depolarization which travels along the nerve In fact it is more than that *since at the point of depolarization a reverse potential is actually* set up *temporarily*

Muscle cells present the same type of polarization across their membranes that nerve cells do The potassium concentration within a frog muscle cell is 20 to 40 times that outside The wave of depolarization, after having passed the length of the nerve and reached the muscle travels along it as well so that during muscular stimulation there is a loss of potassium to the blood (7) which is regained during recovery

Acetylcholine The question of the chemical mechanisms associated with the electrical phenomena involved in nerve conduction is one which has been, and still is being debated hotly The difficulties involved in studying *the chemistry of the minute nerve fibers of man* and other vertebrate species have caused many of the applicable experiments to be performed, of necessity, upon the giant axon of the squid and the degree to which results on squid nerve can be applied to human nerve is always questionable

The transmission of the nerve impulse is accompanied by and perhaps effect [illegible] release of acetylcholine (ACh) (formula XCII) from [illegible] two most direct experiments indicating

this to be so are (a) ACh applied to synaptic regions will stimulate a nerve impulse, and, (b) the appearance of ACh in the perfusion fluid of synaptic regions following nerve stimulation Neither piece of evidence is conclusive, since other substances than ACh have been found to stimulate nerve and the appearance of ACh in the synaptic region takes place only after rather strong stimulation and may be a sign of injury rather than the result of any physiological process



Indirect evidence concerning the functioning of ACh in nerve transmission is derived from studies of *cholinesterase*, an enzyme which catalyzes the hydrolysis of ACh to acetic acid and choline (formula XCIII) The facts about cholinesterase which bear upon the problem may be summarized as follows

(1) Cholinesterase is widely present in nerve and muscle tissue and is always localized at the neuronal surface where the bioelectric phenomena occur This means that cholinesterase tends to be localized at the synaptic junction where the repeated division and subdivision of the axon results in finer fibers and a consequent greater surface-volume ratio

(2) Cholinesterase in nerve and muscle is much more specific than the

factory theory exists to explain the accumulation of potassium against a concentration gradient, but the current concept is that potassium forms a protein complex within the cell (27), thus becoming part of a system to which the cell membrane is impermeable, shifting the equilibrium in favor of continued influx of potassium Such bound potassium is in equilibrium with ionic potassium as isotope exchange experiments show For a fuller discussion of the subject, the student is referred to a review by Sheppard (24)

The difference in concentration between potassium within and without the cell causes a potential difference to exist across the membrane since *the system, nerve cell/surrounding medium, possesses the characteristics of* what is known in electrochemistry as a "concentration cell" A membrane across which such a potential difference exists is known as a *polarized membrane*

The potential difference across the nerve membrane has been found to be between 75 and 95 millivolts The maintenance of such a difference, of course, requires energy which is derived from the metabolism of glucose within the cell A dead nerve cell is unable to maintain the potential difference, and the potassium concentration equalizes across the membrane, which is then said to be *depolarized* If a section of a nerve fiber is injured, that part is depolarized and a potential is set up between it and the remaining uninjured portions A nerve impulse is accompanied by a naturally occurring depolarization which travels along the nerve In fact, it is more than that since at the point of depolarization a reverse potential is actually set up temporarily

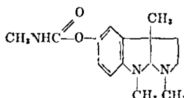
Muscle cells present the same type of polarization across their membranes that nerve cells do The potassium concentration within a frog muscle cell is 20 to 40 times that outside The wave of depolarization, after having passed the length of the nerve and reached the muscle, travels along it as well, so that during muscular stimulation there is a loss of potassium to the blood (7) which is regained during recovery

Acetylcholine. The question of the chemical mechanisms associated with the electrical phenomena involved in nerve conduction is one which has been, and still is, being debated hotly The difficulties involved in studying the chemistry of the minute nerve fibers of man and other vertebrate species have caused many of the applicable experiments to be performed, of necessity, upon the giant axon of the squid and the degree to which results on squid nerve can be applied to human nerve is always questionable

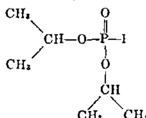
-³ by and perhaps
ula XCII) from
ments indicating

has been established in the electric organ of *Electrophorus electricus*, the electric eel

Not all neurophysiologists and biochemists are satisfied with the ACh hypothesis as presented by Nachmansohn The arguments against it have been summarized by Gerard (8) However those who do not accept the ACh hypothesis have so far been rather negative in their approach They have presented some experimental results which appear to be inconsistent with the ACh theory, but apparently do not have an alternate theory to present Even Gerard is now willing to admit that the ACh mechanism is satisfactory at autonomic effectors and probably at neuromuscular



XCIV Physostigmine



XCV Diisopropylfluorophosphate (DFP)

junctions and autonomic synapses, although he still doubts its function in transmission in the central nervous system and, especially, thinks it is not essential to conduction in peripheral nerve

Not all nerves liberate ACh upon stimulation Stimulation of the sympathetic nerves liberates a substance, earlier called *sympathin*, and later subdivided into sympathin E and sympathin I, the former having been thought associated with the excitatory effects of sympathetic stimulation and the latter with the inhibitory effects Tainter and Luduena (29) favor abandonment of the sympathin terminology The sympathins are actually *adrenalin* and *noradrenalin*, the latter differing from the former in lack of the N methyl group (formulas XCVIa and XCVIb)

Nerve fibers which secrete the sympathins on stimulation are termed *adrenergic fibers*, those which secrete ACh, *cholinergic fibers* The adrenergic fibers include most of the postganglionic sympathetic fibers, while the

average esterase (i e , *ester-hydrolyzing enzyme*) Cholinesterase will catalyze the hydrolysis of ACh and one or two other very closely related choline esters Another esterase exists which can split ACh, *but this catalyzes the* hydrolysis of many other esters It is distinguished from cholinesterase by the name pseudocholinesterase The specificity of cholinesterase is strong evidence for the assumption that its function in nerve tissue is to hydrolyze the undoubtedly present ACh Presumably, after formation of ACh in the process which accompanies, or perhaps initiates, nerve impulse transmission, it must be quickly inactivated to render that portion of the nerve amenable to initiation of another impulse Cholinesterase would be the agent employed in that process ACh could be re formed in the period of nerve recovery through the action of *choline acetylase*, an enzyme which has been isolated from brain tissue Choline acetylase catalyzes the condensation of acetic acid and choline to form ACh Since the hydrolysis of ACh is the spontaneous reaction, going to virtual completion, its re formation *in significant quantities can not take place without the input of con*siderable energy The energy necessary is derived from a high phosphate bond, and choline acetylase will not perform its function except in the presence of ATP, which in the process is hydrolyzed to ADP Also, the pantothenic acid derivative, coenzyme A (22), is necessary for the reaction to proceed

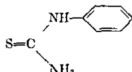
(3) The high concentration of cholinesterase in nerve tissue makes possible the removal of ACh at a speed comparable to that of the electrical phenomena One of the great stumbling blocks to any chemical theory of nerve transmission has been to find a reaction capable of proceeding at such a speed Nachmansohn, working with the frog's sartorius muscle, showed *that 1 6 $\times$ 10⁹ molecules of ACh could be hydrolyzed in one thou*sandth of a second at a single motor end plate This would correspond to the ACh content of 100 to 250 square microns of neuronal surface In mammalian brain, 10^{14} to 10^{15} molecules of ACh may be inactivated per gram of tissue, which would correspond to the ACh content of 10 to 100 square millimeters of neuronal surface

(4) Substances which inhibit cholinesterase alter, and in high concentrations abolish, the nerve action potential Such a substance is *physostigmine* (formula XCIV), an alkaloid of the calabar bean By inhibiting cholinesterase activity, it prolongs and intensifies nerve impulse and acts pharmacologically as a parasympathetic stimulant More recently, di isopropylfluorophosphate (DFP) (formula XCV) has been used experimentally as a reversible cholinesterase inhibitor The principle of cholinesterase *inhibition* is the basis of the so called "nerve gases" which are being spoken of in connection with possible future wars

(5) A direct proportionality between voltage and cholinesterase activity

is apparently subject to Mendelian inheritance and has been consequently studied for its relationship to problems of anthropology

Sight The retina contains chromoproteins usually termed *visual pigments* because of their role in the phenomenon of sight The prosthetic groups of these visual pigments are carotenoid in nature Of these the best known is *rhodopsin* also called erythropsin or visual purple' Its carotenoid content is 10 per cent Upon exposure to light the bond between protein and prosthetic group is broken and the intensity of the color of the complex diminishes markedly for which reason the mixture of protein and free carotenoid is called visual yellow The carotenoid thus liberated is *retinene*, an aldehyde of vitamin A In the dark, regeneration of rhodopsin takes place A continuous supply of vitamin A is apparently necessary for efficient regeneration, perhaps because some of the retinene is lost through decomposition during its free existence Rhodopsin is concerned with vision under faint illumination which is a function of the rods of the retina It is for this reason that avitaminosis A leads to night blindness



XCVII Phenylthiourea

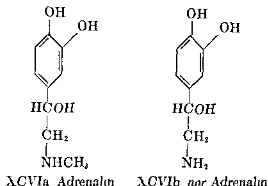
The crystalline lens and the cornea are unique among body solids for their high degree of transparence to light This is not due to any unusual tissue components Both consist almost entirely of water and protein The crystalline lens is unusually high in protein content which amounts to 35 per cent of its weight Some 85 per cent of the protein are *crystallins* which are chiefly remarkable in their low degree of specificity among varying species of animals When an animal is sensitized to the crystallins of a particular species of mammal antibodies are formed which will react with the crystallins of almost any other mammalian species but not with those from birds The crystallins have been divided into two groups alpha and beta the former exhibiting high antigenic power and the latter low The remainder of the crystalline lens protein is albuminoid in nature The cornea contains 18 per cent proteins of which four fifths is collagen and the remainder mucoprotein

The aqueous humor and vitreous humor are intraocular fluids of similar composition which is formed from the blood plasma and contained in the eye under pressure The humors differ from blood plasma in being almost pure water Only a little over one per cent of their weight is solid matter and of this some three fifths is sodium chloride The aqueous humor has

cholinergic fibers include the sympathetic and parasympathetic preganglionic fibers, the somatic motor fibers, the postganglionic parasympathetic fibers, and certain postganglionic sympathetic fibers such as those which innervate the sweat glands Some of the vasodilator fibers are also cholinergic

Chemistry of Sense Perception

Odor and taste. Of the varieties of sense perception available to man, the sense of smell and the sense of taste are known to be primarily chemical in nature Unfortunately, the nature of the reactions involved is as yet little understood It has been suggested that the sensations of odor and taste result from the inhibition by various substances of one or more enzymes present in the taste buds and olfactory mucosa High concentrations



XCVIa *Adrenalin* XCVIb *nor Adrenalin*

of phosphatase have been found in nasal mucosa of rabbits and high concentrations of both phosphatase and esterase in their taste buds There was some evidence to the effect that substances such as vanillin inhibited the phosphatase activity in the taste buds, while quinine inhibited that of the esterase (6) If the mechanism which makes the sensations of taste and odor possible were indeed to involve enzyme inhibition, it would account both for the minute quantities of some substances capable of being smelled or tasted, and for the wide variety of chemicals yielding similar taste or odor, since enzymes can usually be inhibited by many different substances of widely varying nature For information on the classification of tastes and odors and on the effects of individual substances, the student is referred to Moncrieff (18)

The quantity and quality of taste of a given substance can vary from individual to individual Thus, phenylthiourea (formula XCVII) and certain related sulfur containing substances, while bitter in varying degrees to some people are completely tasteless to others (4) Such "taste blindness"

19 MONNÉ, L Functioning of the cytoplasm Advances Enzymol 8 1–69, 1948
20 MUSTACCHI, P O Extensibility and minimum number of polypeptide chains in the collagen micelle Science, 113: 405–407, 1951
21 NACHMANSOHN, D Chemical mechanism of nerve activity Ann New York Acad Sc, 47: 395–428, 1946
22 NACHMANSOHN, D, AND BERMAN, M Studies on choline acetylase III On the preparation of the coenzyme and its effect on the enzyme J Biol Chem, 165. 551–563 1946
23 PIGMAN W W, AND GOEPP R M, JR *Chemistry of the Carbohydrates* Academic Press, New York 1948
24 SHEPPARD, C W New developments in potassium and cell physiology, 1940–1950 Science, 114: 85–91, 1951
25 SHOPPEE, C W Steroid configuration Vitamins & Hormones 8 255–308, 1950
26 SOBOTKA, H *Physiological Chemistry of the Bile* The Williams & Wilkins Co, Baltimore, Md, 1937
27 STONE, D AND SHAPIRO, S Free and bound potassium in rat brain and muscle Am J Physiol, 155 141–146 1948
28 SZENT GYÖRGYI, A *Chemistry of Muscular Contraction* 2nd ed Academic Press, New York, 1951
29 TAINTER, M L, AND LUDUENA F P Sympathetic hormonal transmission Recent Progr Hormone Research 5: 3 36, 1950
30 WEBB, D A, AND YOUNG J Z Electrolyte content and action potential of the giant nerve fibers of *Loligo* J Physiol, 98. 299–313, 1940
31 WILHELMI, A E in FULTON, J F, ed *A Textbook of Physiology* 16th edit, pp 142 176 W B Saunders Co Philadelphia, 1949

in it traces of glucose, glutathione, and ascorbic acid, all of which play a part in the maintenance of the metabolic processes of the crystalline lens, which is, of course, free of any direct vascularization Beta-crystallin may play a role as oxidizing enzyme in this respect

Tears are similar to the humors in chemical composition, possessing in fact a somewhat larger percentage of solid matter (1 8 per cent in tears as compared with 1 1 per cent in the humors) including proteins and glucose as well as the commonly recognized sodium chloride The most interesting component of tears is lysozyme, which, through its lytic action upon bacteria, gives to tears the qualities of a mild disinfectant

REFERENCES

1 Artom, C Some data on the distribution of individual phospholipids in rat tissues and in human plasma J Biol Chem, 157: 595–599, 1945
2 Bailey, K Molecular weight of tropomyosin from rabbit muscle Biochem J, 43: 279–281, 1948
3 [illegible]
4 [illegible] Psychol Bull 48. 71–74, 1951
5 Conway, E J, and Hingerty, D The influence of adrenalectomy on muscle constituents Biochem J, 40: 561–568, 1946
6 El Baradi, A F, and Bourne, G H Theory of tastes and odors Science, 113: 660–661, 1951
7 Fenn, W O, and Cobb, D M Electrolyte changes in muscle during activity Am J Physiol, 115: 345–356, 1936
8 Gerard, R W The acetylcholine system in neural function Recent Progr Hormone Research, 5: 37–65, 1950
9 Graham, C E, et al The amino acid content of some scleroproteins J Biol Chem, 177. 529–532, 1949
[illegible] Wiley
[illegible] of fat
[illegible]
17 Mommaerts, [illegible] York, 1950
18 Moncrieff, R W The Chemical Senses John Wiley & Sons, Inc, New York, 1946

THE CLOTTING OF THE BLOOD

When blood escapes from the blood vessels, it ordinarily soon solidifies into a gelatinous mass called the clot Clotting is one of the mechanisms involved in hemostasis which occurs in the following stages (12)

1 Vessels are severed by injury, and become dilated by the histamine liberated as the result of tissue damage Blood therefore flows from the wound

2 After a short time the vessels contract from diminished intravascular pressure and from their elastic retraction Blood ceases to flow and now has time to clot

3 After the period of contraction (0 5 to 2 hours), the vessels again dilate, a first step toward wound repair Recurrence of bleeding is prevented by the already formed clot

Abnormal bleeding can be due to (a) failure of initial vascular contraction, (b) failure of clotting or (c) failure of both The framework of the clot consists of *fibrin*, a protein which has a fibrous structure which accounts for its name A network mass of fibrin traps the cells of the blood, and if the blood is normal and the injury not too extensive, bleeding is checked and tissue repair eventually begins on the basis of the blood clot The fibrin itself accounts for less than one per cent of the entire clot

Fibrin is formed from its precursor *fibrinogen*, which occurs in blood plasma Fibrinogen is a globulin, its solutions are highly viscous and show high birefringence of flow The molecules are elongated ellipsoids about 70 millimicrons long, with an axial ratio of 18 to 1 It is formed exclusively in the liver A normal animal "defibrinogenated" by first removing and then replacing blood after the fibrinogen has been removed by whipping will restore the fibrinogen within a few hours replacement of fibrinogen does not occur in the hepatectomized animal In poisoning with substances such as chloroform or arsenic which damage the liver there is often fibrinogen deficiency Congenital *afibrinogenemia* is a rare abnormality characterized by *unclottable blood Fibrinogen may be increased during acute infections* suppurative processes and after hemorrhage

The substance which interacts with fibrinogen converting it to fibrin, is called *thrombin* It is not generally agreed whether or not the conversion of fibrinogen to fibrin is a catalytic reaction, and the sort of alteration which takes place in the fibrinogen molecule is still obscure The circulating precursor of thrombin is called *prothrombin*, and is a protein occurring in the globulin fraction and containing 4 per cent carbohydrate Thrombin of course does not occur in circulating blood, since its presence would cause coagulation Thrombin can be obtained by extraction of fibrin clots, or of alcohol-coagulated serum proteins with dilute salt solutions Thrombin

CHAPTER 4

Blood and the Anemias

Although it is a liquid, blood may be regarded as a tissue If we wish to summarize briefly the usefulness of blood to the other tissues, we may say that it combines in itself the functions of transport and protection, that is, it carries substances to and from the various tissue cells, it protects the tissue cells from abrupt and undesirable changes in the interstitial fluid which is their immediate environment, and it protects them against invasion by foreign organisms

The blood is the chief medium of distribution of nutrients such as monosaccharides and amino acids to the various tissues It also brings to the tissues the oxygen without which energy producing substances could not be utilized fully, takes away from the tissues waste products such as carbon dioxide, urea, uric acid, and conducts away the excess heat so that it can be radiated from the surface of the body The blood also carries hormones from one tissue to another and thus mediates the control exercised upon physiological function by the various endocrine glands It also conveys antibodies to fight infection, and helps bring phagocytes to the site of an invasion so that they may devour the micro organisms attempting to establish a foothold

In addition to transport, the blood has the function of protecting the various tissues It does this in various ways In addition to the transport of antibodies and phagocytes, it supplies to all of the tissues a fluid of suitable osmotic pressure for correct function By means of its buffer power it protects the tissues against changes in hydrogen ion concentration which might impair their efficiency In order that the volume of blood may be maintained relatively adequate, there are two necessary additional functions which the blood must have The first of these involves the clotting mechanism, which restricts loss of blood A second exists, whereby water can be drawn into the circulation so that the plasma is kept suitably dilute and the total blood volume at a suitable level This osmotic function also enables blood to mobilize water from tissues in case of emergency

agent because it acts as an antagonist to vitamin K It is used to prevent thrombosis (intravascular clotting) in man as in patients following operation, but its action is delayed 24 hours or more and it is therefore useless in emergencies

A substance which does act immediately is *heparin* It is generally distributed throughout the body but is most concentrated in the liver and lungs It is a mucopolysaccharide containing mucoitin sulfuric acid Heparin plus a cofactor which comes from the plasma can act as an anticoagulant, since it has properties of antithrombin, an antithromboplastin and an antiprothrombin Purified preparations of heparin are used to render the blood promptly incoagulable *in vivo*

The reaction between prothrombin and thromboplastin appears to be stoichiometric, since if small amounts of prothrombin are used in the presence of adequate amounts of thromboplastin doubling the amount of prothrombin doubles the yield of thrombin (18a)

Hemophilia is a hereditary sex linked disease (see Chapter 9 characterized by a lifelong tendency to prolonged hemorrhage even after slight injury It is so rare that up to the present time it has been reported only in males, although theoretically females with two chromosomes containing this gene could exhibit the disease, providing they could survive to an age which would allow them to be observed

The fundamental abnormality is that the coagulation time of the blood is greatly delayed There has been much dispute as to the cause of this It has been shown that it is not due to a lack of fibrinogen or calcium, nor are the platelets reduced in number Some have supposed that the platelets were less fragile than normal platelets and therefore liberated thromboplastin less promptly Even this is not fully established There is a small amount of a globulin in normal blood which Taylor has referred to as "antihemophilic globulin", and which appears to be lacking in cases of hemophilia (4)

THE BUFFER POWER OF THE BLOOD

Since most of the plasma proteins have isoelectric points distinctly acid to ordinary physiological pH most of these proteins are behaving under physiological conditions as anions We may regard the blood proteins and their salts together as constituting a mixture of a weak acid and its salt with a strong base which constitutes a typical buffer system (see Chapter 16) In addition to the protein protein salt buffer systems in the blood, we have other systems such as the dissolved carbonic acid and sodium bicarbonate and mixtures of phosphates Also the combination of oxygen with hemoglobin increases its affinity for base The net result is that the blood possesses considerable buffer power

We may represent the buffers of the blood as shown in formula I—where

preparations of human, beef, and rabbit origin are available, and are used by surgeons in local hemostasis Hirudin an antithrombin from the leech, will prevent thrombin action

Prothrombin is very unstable in aqueous solution, being damaged by heat at temperatures as low as 36°C In the dry form, however, it may be stable for years Its electrophoretic mobility is higher than that of other plasma proteins It has been estimated that normal human plasma contains less than 20 mgm of prothrombin per 100 ml of blood, but nevertheless this low concentration exerts a powerful action Plasma can be diluted three hundred times and still, when the prothrombin is fully activated, show a clotting effect upon fibrinogen solutions

Prothrombin deficiency may result from liver disease, since the liver is the exclusive site of prothrombin formation, or from vitamin K deficiency which is most commonly a defect of absorption except in the newborn, in whom vitamin K production by intestinal bacteria has not yet started In the absence of the K vitamins (see page 715) the liver is unable to make prothrombin The conversion of prothrombin to thrombin involves at least three substances thromboplastin, Ca^{++}, and the plasma accelerator globulin

Thromboplastin[1] is generally distributed throughout the tissues of the body in the blood it or an activator is concentrated in the platelets, as laboratory sources, lung and brain extract prove very satisfactory (17) Calcium ion is necessary for the interaction of prothrombin and thrombo plastin Therefore any agent which precipitates Ca^{++} or binds it in non ionized form will prevent the formation of thrombin This explains the use of substances such as oxalate and citrate as anticoagulants Ion exchange resins which remove Ca^{++} are also used

Since Ca^{++} is always present in the blood, it is obvious that active throm boplastin can not exist free in the plasma under ordinary conditions as otherwise thrombin would be formed which would convert the fibrinogen into fibrin and result in intravascular clotting It is generally supposed that thromboplastin or an activator is liberated from the blood platelets when they are damaged by adhering to foreign surfaces, and precautions such as the use of oil oiled syringes plastic polyethylene containers, or tubes lined with silicone or paraffin wax will retard clotting There is also evidence that thromboplastin exists in an inactive form, dissolved in the plasma and is activated by contact with foreign surfaces or by a substance liberated by blood platelets

3,3 Methylene bishydroxycoumarin, commonly called dicumarol, has been shown to be the causative agent in a hemorrhagic disease of cattle caused by ingestion of improperly cured sweet clover It is an anticlotting

meaning In one sense it is a thermodynamic function calculated from the concentration of dissolved particles present In another sense it is the pressure which must be exerted on the solution to prevent the entrance of additional solvent through a semi permeable membrane with which the solution is in contact Thus, the colloid osmotic pressure acts to draw water into the blood and to maintain a proper volume of circulating fluid

As we shall see later the proteins of the blood plasma may be roughly divided into two classes—albumin and the globulins Serum albumin has a molecular weight of about 70 000 Most of the serum globulins have molecular weights of the order of 160 000 It will be recalled that the osmotic pressure depends on the number of dissolved particles per unit volume It will therefore be appreciated that a one per cent solution of serum albumin will have more than twice the effect on the colloid osmotic pressure than will a one per cent solution of serum globulin There is about twice as much albumin present as globulin and as far as our present knowledge goes, the osmotic activity of serum albumin is its chief function

Another factor which increases the importance of serum albumin in maintaining the osmotic pressure of the blood is based on the fact that it has a lower isoelectric point than globulins Consequently at physiological pH it carries a higher negative charge, and thus is associated with a larger number of cations such as Na^+ These cations function also as particles in determining osmotic pressure and although the walls of the capillaries are permeable to such ions, enough cations must remain in the blood to neutralize the negative charges on the protein molecules It has been found that one gram of serum albumin holds about 18 ml of water in the blood This figure is arrived at both from clinical experiments following the injection of concentrated solutions and laboratory experiments on the osmotic pressure of purified albumin

Although some, perhaps all of the globulins have functions of importance other than maintenance of osmotic pressure they also act as negatively charged colloid ions and contribute about 20 per cent of the colloid osmotic pressure of the blood

The importance of the osmotic function of the plasma proteins is clearly shown when there has been an acute loss of blood Even before the amount of blood lost is sufficient to lower the oxygen and carbon dioxide transporting power seriously, grave results are observed and in severe cases the patient goes into shock (see Chapter 15)

In conditions of shock resulting from an acute loss of plasma proteins the introduction of human plasma has been extensively employed and is of great value The plasma was originally obtained by centrifuging off the formed elements of blood to which citrate had been added as an antico-

B equals a cation such as sodium Hb equals hemoglobin, and Pr equals protein

As a result of this buffer capacity and the excretion of CO_2 by the lungs and of acids or bases in the urine (see page 654), the pH of blood does not normally vary from 7 4 The maximum tolerated pH range of blood has been estimated by Van Slyke as from 7 8 to 7 0

It is of interest to notice that when blood is at pH 7 4, the ratio $BHCO_3$ to H_2CO_3 is 20 to 1 and from what we have learned of buffers, we see that the bicarbonate buffer in the blood is not acting at its maximum efficiency Maximum efficiency in buffer systems is attained when the ratio of the acids to the salts equals 1 In the case where the ratio $BHCO_3$ to H_2CO_3 equals 1, the pH is equal to 6 1, this is indeed far from the normal pH of 7 4 It is also of interest to note that it is on the acid side, so that if there is any tendency to shift the pH of the blood in an acid direction, the efficiency of the bicarbonate system becomes greater and greater as this shift goes on, which is fortunate for the animal body With a phosphate buffer the same improvement is found, the situation where the ratio

$$\frac{H_2CO_3}{BHCO_3} \qquad \frac{BH_2PO_4}{B_2HPO_4} \qquad \frac{HHbO_2}{BHbO_2} \qquad \frac{HHb}{BHb} \qquad \frac{HPr}{BPr}$$

I

B_2HPO_4 to BH_2PO_4 equals 1 gives a pH of 6 8 As far as the oxyhemoglobin, which we shall speak of later, is concerned, its maximum buffering effect is at pH 7 7 which is closer to the normal pH of the blood In both directions, then, we see there is considerable protection against changes from physiological pH in the blood The blood, by its rapid circulation, protects tissues from disadvantageous changes in pH We shall have more to say about the buffering capacity of the blood when we come to discuss the transport of oxygen and carbon dioxide (see Chapter 16)

THE OSMOTIC PRESSURE OF THE BLOOD

The hydrostatic pressure in the capillaries, which is produced by the contractions of the heart, tends to expel fluids from the blood, and this is prevented by the osmotic pressure of the plasma proteins which has the effect of drawing water into the blood from the tissue spaces The walls of the capillaries are permeable to small ions such as Na^+, Cl^- and HCO_3^-, so there is no differential osmotic effect due to these ions It is the larger molecules particularly the proteins, of the blood which are important in maintaining the volume of the circulation The osmotic pressure due to these molecules is often called the colloid osmotic pressure This reminds us again that the osmotic pressure of a solution can have more than one

for nitrogen determination whereas with precipitates from ammonium sulfate, dialysis was necessary to remove the NH_4^+, and in some cases doubt remained that it had all been removed The majority of the data available on plasma protein changes in states of disease were until very recently obtained by the method of Howe

In spite of the wide use of the method, it was early apparent that Howe s separation into components was exceedingly crude, and the precipitates overlapped in their content of individual proteins This has since been shown by electrophoretic analysis of precipitates obtained by the Howe technique A further difficulty involved in the Howe method was the uncertainty of the factor for the conversion of the nitrogen to protein The nitrogen figures were multiplied by the conventional 6 25 which, as has been stated previously, was based on the assumption that the average nitrogen percentage of protein is exactly 16 The average nitrogen factor found experimentally in dried proteins from pooled normal human plasma was 6 3 The conversion factor of the individual proteins however, varied from 6 1 to 8 4

In spite of the deficiencies of the Howe method, results of definite clinical usefulness were obtained The total serum protein according to the Howe technique varied from 6 5 to 7 5 grams per 100 ml of blood with a mean of 7 2 grams The mean for the albumin value was 5 2 grams and for the total globulins, 2 0 grams An average A/G ratio therefore was about 2 6 It is obvious that either lowering of the serum albumin content or an increase of the serum globulin content, the first of which might occur in nephrosis for example and the latter in many types of infection would give a decreased A/G ratio However, such a change could result from a combined decline in albumin and rise in globulin such as is found in hepatic cirrhosis Obviously the single ratio was not adequate and it is preferable to give the absolute values of the serum albumin and serum globulin

Ammonium and sodium sulfates are, of course by no means the only protein precipitants and studies with various others were reported from time to time In particular, it had long been known that ethyl alcohol although at room temperature and ordinary concentrations a protein denaturant, might be used to precipitate plasma proteins if the addition were made carefully at low temperatures Felton had made use of this procedure to separate the antibodies from horse serum

During World War II, Cohn and collaborators developed large scale procedures for the low temperature separation of plasma fractions by the addition of ethyl alcohol They pointed out that ethyl alcohol offers some advantages over salting out by the older methods In the first place, since the alcohol is non ionized one may control the ionic strength of the mixture by the addition of suitable amounts of salts Also by the addition of

agulant Later it was demonstrated that this plasma could be dried from the frozen state and after being redissolved in the proper amount of distilled water would still carry out its osmotic function

Since the albumin of plasma accounts for about 80 per cent of the colloid osmotic pressure, it would be expected that human serum albumin would be effective in combating shock This was found to be true The unit of serum albumin was established as 100 ml of a 25 per cent solution, and it may be computed from the preceding figures that this represents the osmotic equivalent of about 500 ml of citrated plasma

It was observed that patients usually responded almost immediately to the injection of concentrated albumin with a fall in hemoglobin concentration and hematocrit readings and a subsequent decrease in serum protein concentration, indicating that extravascular water had been transferred into the circulation In cases in which the patient was markedly dehydrated, it was found best also to administer saline solution to provide the necessary fluid

Serum albumin has been found useful in combating edema which is due, at least in some cases, to loss of plasma protein particularly of the serum albumin which is osmotically the most effective It is an old clinical observation that when a great deal of albumin has been lost by albuminuria and the plasma proteins are low, proportional degrees of edema are observed The most effective preparations of serum albumin for this purpose are those containing a relatively low concentration of salts

PLASMA PROTEIN FRACTIONATIONS

Blood plasma contains a very complicated mixture of proteins which, out of necessity, we deal with in groups The traditional method of separation of plasma proteins, as with other proteins, was fractional salting out with ammonium sulfate It was found that if plasma was about 20 to 25 per cent saturated with ammonium sulfate, the fibrinogen was precipitated One third saturation (33 3 per cent) separated out a fraction which was called the euglobulin fraction because it would not redissolve in distilled water and thus behaved as a true globulin Further increase in the concentration of ammonium sulfate to half saturation (50 per cent) removed the pseudoglobulin fractions which were soluble in distilled water The remaining protein was called the albumin, and was nearly completely precipitated if the supernatant containing it was brought to full saturation with ammonium sulfate The fractions so obtained were not pure and were capable of further separation

In 1921, Howe (8) published a method which has been widely used clinically Howe used sodium sulfate instead of ammonium sulfate, since in this way his precipitate could be directly digested in the Kjeldahl procedure

Methods of ethyl alcohol fractionation did not produce pure components of plasma, although by re working some of the fractions substantially pure proteins could be obtained—notably in the case of serum albumin and gamma globulin Some success was achieved in purifying the anti A and anti B isoagglutinins (see page 18)

Pillemer and Hutchinson (16) revived the use of methyl alcohol in the fractionation of serum proteins They found that at a concentration of 42 5 per cent methanol at pH 6 7 to 6 9, ionic strength about 0 03 and

TABLE 4

Protein components of human plasma thus far obtained in relatively homogeneous state

ELECTROPHORETIC CLASS	APPROX CONC. IN PLASMA	APPROX MOLECULAR WEIGHT
	g ams/100 ml	
Albumin	3 2	69,000
α_1 globulin	0 2	200,000
α_2 globulin	0 1	300,000
β_1 globulin	0 2	90,000
β_1 globulin	0 2	150,000
β_1 globulin	0 1	500 000-1 000 000
β_1 globulin	0 2	1 300 000
β_2 globulin	0 2	150 000
γ globulin	0 5	156 000
γ globulin	0 1	300 000
Fibrinogen	0 2	400 000

Proteins (except for fibrinogen) arranged in order of decreasing electrophoretic mobility at pH 8 Thus albumin moves faster than α globulin, α globulin moves faster than β globulin, etc Not all plasma proteins have been obtained in as pure a form as those listed here consequently the values given here do not add up to the total value for proteins in plasma

temperature about 0°C, the globulins are almost quantitatively precipitated while almost all the albumin remains in solution The agreement was within 5 per cent with electrophoretic data for normal sera, and for abnormal sera, within 5 to 10 per cent The albumin values obtained were consistently and considerably lower than those derived by the older Howe method, being around 70 to 80 per cent of the Howe albumin levels

The protein components of normal human plasma which have thus far been obtained in a relatively homogeneous state are set forth in table 4 (14)

Electrophoresis and ultracentrifugation have been used to characterize plasma proteins They have the advantage of giving relatively sharp values but are limited by the fact that each depends essentially on a single property of the protein For instance, electrophoresis depends upon the mobility

suitable buffers, the pH, a very important factor in protein fractionation, may be controlled The presence of alcohol permitted the use of temperatures below the freezing point of water

The details of the process and results of the low temperature, low salt ethanol fractionation have been reviewed by Edsall (4) We may summarize them briefly here The fractions were designated by Roman numbers Originally two of the separate fractions were distinguished as II and III, but as these were later found to be very similar chemically, this procedure was abandoned and a combined II + III was removed

Fraction I was obtained by precipitation with 8 to 10 per cent of ethanol by volume at pH 7 2—in other words, the usual pH of pooled citrated plasma—ionic strength 0 14 at a temperature of −3°C which was very close to the freezing point of this mixture This fraction contained about 60 to 65 per cent fibrinogen In combination with thrombin preparations, it found considerable use as a hemostatic agent Another component of this fraction is antihemophilic globulin (see page 167)

Fraction II + III was obtained from the supernatant of Fraction I by raising the alcohol concentration to 25 per cent, changing the pH to 6 8, the ionic strength to 0 09, and the temperature to −5°C This contained virtually all of the gamma globulins, prothrombin, and most of the iso agglutinins (see p 184) It also contains plasminogen, (the inactive pre cursor of plasmin, the proteolytic enzyme of plasma), the midpiece of complement (C'_1) (see page 729), a large proportion of the beta globulins including β_1 lipoprotein which has been called x protein by Macfarlane, and two other distinct β_1 globulins, a β_2 globulin, and some fibrinogen and antihemophilic globulin not removed in Fraction I

Fraction IV, which was usually further subdivided, appeared as Fraction IV-1 which was obtained from the supernatant of II + III by lowering the ethanol concentration to 18 per cent and the pH to 5 2 It contained a large proportion of the alpha globulin, mostly as an α_1 lipoprotein containing about 35 per cent of lipids, and in addition a blue green pigment and other components Fraction IV-4 obtained from the supernatant of IV 1 by increasing the ethanol concentration to 40 per cent by volume and the pH to 5 8, contained nearly lipid free alpha and beta globulins and some albumin It contained an esterase and a β_1 globulin of relatively low molecular weight

Fraction V was obtained from the supernatant of Fraction IV 4 by lowering the pH to 4 8 with acetate buffer so as to give an ionic strength of 0 11 while maintaining the ethanol concentration at 40 per cent It was separated at a temperature of −5°C, and was composed almost entirely of albumin The supernatant of Fraction V contained less than 2 per cent of the total protein and was made up mostly of albumin and alpha globulin

represents a 1 10 dilution of the original blood This enables values for blood to be calculated easily from the figures obtained Some of the reducing power of the blood filtrate is not due directly to glucose but to non-sugar reducing substances They may amount to as much as 20 to 30 per cent of the total Since, however they seem to remain fairly constant, it has proved clinically satisfactory to continue to estimate all of the reducing substances as glucose, and the rough rule that a normal value is not far from 100 mgm per 100 ml of blood is convenient to remember Much higher values of the order of hundreds of milligrams may be seen in diabetic patients

The total lipids of blood plasma were found to be 507 ± 73 (on a series of 21 subjects) (9) The components of the blood lipids will be discussed in Chapter 13 Hormones vitamins organic acids, and phenols are present in the blood in small amounts In diabetes and starvation appreciable amounts of acetone and acetoacetic acid may occur The color of the plasma is chiefly due to bilirubin, with a minor contribution of carotenoids

The nitrogenous constituents of the blood for the most part represent waste products on their way to the kidney to be excreted The total *non protein nitrogen* (NPN) may be estimated by doing a Kjeldahl digestion of a measured amount of blood filtrate and estimating the nitrogen present Normal values range from 25 to 35 mgm N per 100 ml blood but higher values are found in some diseases such as nephritis and eclampsia

The largest component of the non protein nitrogen of the blood is the *urea* nitrogen which normally amounts to 10 to 15 mgm of nitrogen per 100 ml of blood The value may be higher in chronic or acute nephritis, metallic poisoning which affects kidney function, cardiac failure prostatic obstruction and in some infectious diseases

It would have been desirable perhaps to express all of the other non protein nitrogenous constituents of the blood in terms of nitrogen but unfortunately, since they are usually determined by reactions not directly dependent upon their nitrogen content, they are usually expressed in terms of milligrams of the substance itself per 100 ml of blood To understand clinical literature, the student should be familiar with these values From a knowledge of the formulas and atomic weights of the elements composing them the values given can be converted if desired into nitrogen

Uric acid occurs to the extent of 2 to 3 5 mgm per 100 ml of normal blood It is a waste product and is excreted in the urine Values higher than normal may be found in nephritis eclampsia and in gout, a disease in which uric acid or its salts are deposited in crystalline form in the cartilages, especially of the joints

Creatinine occurs to the extent of 1 to 2 mgm per 100 ml Its value may be increased in severe nephritis *Creatine*, of which creatinine is the an

in an electric field of the protein at the pH used, and we know that some plasma proteins, although very different in molecular size, shape, chemical constitution, and biological significance happen to have the same or very similar mobilities and thus appear together as an apparently homogeneous peak It is for this reason that with few exceptions the numerous antibodies contained in human plasma have not been separated from the other gamma globulins When the electrophoresis method was first introduced it was hoped that different and specific electrophoretic spectra would characterize different diseases We now know that this is not in general the case, but the changes follow certain common patterns much as in the case of sodium sulfate fractions For instance, a decrease in serum albumin is associated with many kinds of disease, especially those accompanied by wasting or malnutrition or an acute febrile illness This decrease is especially pronounced when albumin is being lost in the urine as in nephrosis, or by exudation through denuded surfaces as in burns, or in disturbances in albumin formation such as cirrhosis of the liver There is also a characteristic increase in the gamma globulin (which includes the antibodies) in various infections, in cirrhosis, and in many cases of multiple myeloma (see page 668) as was already known from the studies based on the Howe method In general there is a rough sort of parallelism between results of the two methods

Ultracentrifugation has not been applied very much to the study of pathological sera Some interesting results have been obtained in multiple myeloma, where efforts were made to identify Bence-Jones protein in the blood and urine A summary of plasma protein changes in various diseases will be found in the review by Gutman (6)

NON-PROTEIN ORGANIC CONSTITUENTS

Remembering that one of the chief functions of blood is transport, it is not surprising to find that most of the non protein constituents are either substances going on their way to the tissues from the point at which they were absorbed, traveling from one tissue to another, or going from the tissues as waste products to be excreted It is customary to discuss these in several groups The most important non nitrogenous organic constituent is *glucose* It is determined by methods which depend upon its power of reducing metallic ions such as Cu^{++}, and it is generally stated that the normal values vary from 80 to 120 mgm of glucose per 100 ml of blood The method of Folin and Wu involves laking the blood—that is, adding a sufficient amount of water to cause the red corpuscles to break up and liberate their contents then a precipitation of all the proteins by formation of tungstic acid by the addition first of sodium tungstate and then of sulfuric acid The volumes used are adjusted so the resulting blood filtrate

The Red Cells (Erythrocytes)

The red cells are the most numerous cells in the blood and their function is well understood Mature erythrocytes are composed almost entirely of hemoglobin, water, and stroma The stroma is often spoken of as merely the cell membrane, but there is some evidence that it permeates the entire cell This is supported by observation For instance, erythrocytes can be partially hemolyzed, that is when immersed in mildly hypotonic solutions such that the osmotic pressure allows water to enter and rupture the cell membrane not all the hemoglobin is lost If restored to an isotonic solution a large number of gradations between ordinary cells and completely hemolyzed cells commonly referred to as ghosts may be obtained The stroma may be obtained after hemolyzing the corpuscles with distilled water It is found to be rich in phospholipid and cholesterol These constitute about 20 per cent or more of the dry weight of the stroma There are also inorganic materials, and proteins peculiar to the erythrocyte envelope The stroma

TABLI 5

Average concentrations of sodium in serum and red cells in mFq liter

	SODIUM	POTASSIUM
Serum	137	4 4
Cells	21	95 1

also contains the numerous blood group substances which will be discussed later in this chapter The red corpuscles of man and most animals tend to accumulate K^+ preferentially to Na^+, so that there is a characteristic difference in the concentration of the two ions within and without the cell This is often spoken of as differential permeability of the cell membrane It is more likely, however, that it is the result of the action of the living cell in accepting certain ions and rejecting others The table of measurements on rabbit blood (table 5) will serve to illustrate the difference observed in the content of potassium and sodium

Thus, we see potassium is twenty times as concentrated in the corpuscles as in the plasma

Hemoglobin

The main protein contained in the red cells of the blood is hemoglobin It is a conjugated protein made up of a porphyrin derivative, heme, as the prosthetic group of the basic protein, globin usually classified as a histone The iron containing prosthetic group has been synthesized and successfully coupled to the protein portion, globin Hemoglobin contains 0 335 per cent iron If we assume one atom of iron (atomic weight 55 0) per molecule, this

hydride, occurs in larger amounts, 3 to 7 mgm, but *is not ordinarily* determined since it lacks clinical significance The *amino acid nitrogen* of the various amino acids on their way to and from the tissues occurs to the extent of 5 to 8 mgm of nitrogen per 100 ml This may be increased in leukemia, diseases involving damage to the liver, and in severe nephritis

Ammonia nitrogen does not occur in the blood in significant amounts Some investigators have reported values of ammonia nitrogen ranging from 0 1 to 0 2 mgm N per 100 ml in normal blood The ammonium ion *is rapidly converted to urea in the liver* (see Chapter 14) By this means the concentration of the quite toxic ammonia is kept at very low levels in the blood

INORGANIC CONSTITUENTS

The sodium ion occurs mostly in the plasma, the potassium ion mostly in the erythrocytes Plasma Cl^- occurs to the extent of 100 to 106 mEq per liter Sulfates of the blood are usually estimated as inorganic sulfate in the serum Values ranging from 0 6 to 1 5 mEq per liter are encountered, they may be somewhat higher in nephritis Phosphate, estimated as inorganic phosphorus of plasma, occurs to the extent of 3 to 4 5 mgm of P per 100 ml Normal values are somewhat higher *in children Phosphate may* be increased in nephritis and may be low in rickets Calcium occurs in the serum to the extent of 9 0 to 10 5 mgm per 100 ml (4 5 to 5 3 mEq per liter) Low values are encountered in infantile tetany, severe nephritis and following removal of or damage to the parathyroids Magnesium occurs in serum to the extent of 1 5 to 2 5 mEq per liter Sodium ion occurs in the serum to the extent of about 136 to 145 mEq per liter Potassium in *the serum ranges from 3 5 to 5 0 mEq per liter Iodine occurs to the extent* of 3 5 to 7 5 micrograms per liter

The plasma contains numerous enzymes, of which the following are of clinical importance amylase, lipase, acid phosphatase, and alkaline phosphatase

THE FORMED ELEMENTS OF THE BLOOD

Within the blood plasma floats a number of cells which have a definite *structure and a definite form, although some of them are plastic and can* admit to a *considerable amount of deformation* These are known as the formed elements of the blood, they amount to about 42 to 50 per cent by volume of the normal blood of the male, 40 to 48 per cent for the *female* The form [illegible] and leukocytes The chemical [illegible] from that of tissue [illegible] capable of phagocytosis, an important function of the blood [illegible] chief defense against microbial invasion

The Red Cells (Erythrocytes)

The red cells are the most numerous cells in the blood and their function is well understood Mature erythrocytes are composed almost entirely of hemoglobin, water, and stroma The stroma is often spoken of as merely the cell membrane, but there is some evidence that it permeates the entire cell This is supported by observation For instance, erythrocytes can be partially hemolyzed, that is when immersed in mildly hypotonic solutions such that the osmotic pressure allows water to enter and rupture the cell membrane, not all the hemoglobin is lost If restored to an isotonic solution a large number of gradations between ordinary cells and completely hemolyzed cells commonly referred to as ghosts may be obtained The stroma may be obtained after hemolyzing the corpuscles with distilled water It is found to be rich in phospholipid and cholesterol These constitute about 20 per cent or more of the dry weight of the stroma There are also inorganic materials, and proteins peculiar to the erythrocyte envelope The stroma

TABLE 5

Average concentrations of sodium in serum and red cells in mEq /liter

	SODIUM	POTASSIUM
Serum	137	4 4
Cells	21	95 1

also contains the numerous blood group substances which will be discussed later in this chapter The red corpuscles of man and most animals tend to accumulate K^+ preferentially to Na^+, so that there is a characteristic difference in the concentration of the two ions within and without the cell This is often spoken of as differential permeability of the cell membrane It is more likely however, that it is the result of the action of the living cell in accepting certain ions and rejecting others The table of measurements on rabbit blood (table 5) will serve to illustrate the difference observed in the content of potassium and sodium

Thus, we see potassium is twenty times as concentrated in the corpuscles as in the plasma

Hemoglobin

The main protein contained in the red cells of the blood is hemoglobin It is a conjugated protein made up of a porphyrin derivative, heme, as the prosthetic group of the basic protein, globin usually classified as a histone The iron containing prosthetic group has been synthesized and successfully coupled to the protein portion, globin Hemoglobin contains 0 335 per cent iron If we assume one atom of iron (atomic weight 55 9) per molecule, this

hydride, occurs in larger amounts, 3 to 7 mgm, but is not ordinarily determined since it lacks clinical significance The *amino acid nitrogen* of the various amino acids on their way to and from the tissues occurs to the extent of 5 to 8 mgm of nitrogen per 100 ml This may be increased in leukemia, diseases involving damage to the liver, and in severe nephritis

Ammonia nitrogen does not occur in the blood in significant amounts Some investigators have reported values of ammonia nitrogen ranging from 0 1 to 0 2 mgm N per 100 ml in normal blood The ammonium ion is rapidly converted to urea in the liver (see Chapter 14) By this means the concentration of the quite toxic ammonia is kept at very low levels in the blood

INORGANIC CONSTITUENTS

The sodium ion occurs mostly in the plasma, the potassium ion mostly in the erythrocytes Plasma Cl^- occurs to the extent of 100 to 106 mEq per liter Sulfates of the blood are usually estimated as inorganic sulfate in the serum Values ranging from 0 6 to 1 5 mEq per liter are encountered, they may be somewhat higher in nephritis Phosphate, estimated as inorganic phosphorus of plasma, occurs to the extent of 3 to 4 5 mgm of P per 100 ml Normal values are somewhat higher in children Phosphate may be increased in nephritis and may be low in rickets Calcium occurs in the serum to the extent of 9 0 to 10 5 mgm per 100 ml (4 5 to 5 3 mEq per liter) Low values are encountered in infantile tetany, severe nephritis and following removal of or damage to the parathyroids Magnesium occurs in serum to the extent of 1 5 to 2 5 mEq per liter Sodium ion occurs in the serum to the extent of about 136 to 145 mEq per liter Potassium in the serum ranges from 3 5 to 5 0 mEq per liter Iodine occurs to the extent of 3 5 to 7 5 micrograms per liter

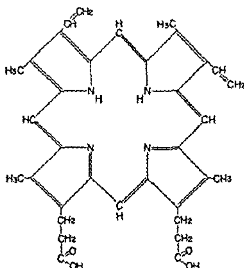
The plasma contains numerous enzymes, of which the following are of clinical importance amylase, lipase, acid phosphatase, and alkaline phosphatase

THE FORMED ELEMENTS OF THE BLOOD

Within the blood plasma floats a number of cells which have a definite structure and a definite form, although some of them are plastic and can admit to a considerable amount of deformation These are known as the formed elements of the blood, they amount to about 42 to 50 per cent by volume of the normal blood of the male, 40 to 48 per cent for the female The formed elements consist mainly of erythrocytes and leukocytes The chemical constitution of leukocytes does not differ significantly from that of tissue cells in general Some leukocytes are motile, and are capable of phagocytosis, an important function of the blood and the chief defense against microbial invasion

moglobin

r of different nomenclatures for hemoglobin and its de
n proposed here we have chosen to follow the nomen
erg and Legge (11) The iron in hemoglobin even when
is in combination with oxygen is in the ferrous state and
magnetic susceptibility shows that the compound is diamag
he iron bonds are therefore covalent The oxygen molecule
ns two unpaired electrons and it is evident that it has



II

oundly by combining with hemoglobin and the system reso
two structures A and B where the electrons in the third
epresented by dots (formula V) If the iron of hemo
the ferric state, we then have the compound called
n, and by others *methemoglobin* which no longer has

g

er oded animals make use of hemoglobin
and certain lower forms have red heme
lar weight possessing iron Other lower
lecule which contains copper but not
called *hemocyanins* Some snails have
ers have iron-containing compounds

percentage leads to a minimum molecular weight of about 16,000 As in many other examples of calculation of minimal molecular weight, it turns out that this value is too small Osmotic pressure and ultracentrifugal measurements show that in fact the molecular weight of hemoglobin is about 68,000, which shows there must be four atoms of iron per molecule Hemoglobin has the property of combining reversibly with oxygen, and it is this property which makes it possible for oxygen to be transferred in adequate amounts from the lungs to the tissues If the absorption spectrum of *oxyhemoglobin*, that is, hemoglobin combined with oxygen, is examined, it shows two sharp absorption bands in the green, leaving the transmitted light which we see a bright red Dilute solutions of oxyhemoglobin have a yellowish tinge Examination of red cells with high magnification under the microscope demonstrates the same yellow color

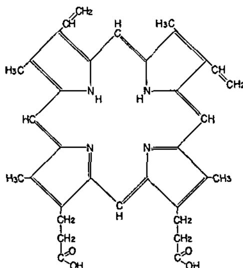
Porphyrins

The iron containing portion of hemoglobin has been found to be a porphyrin combined with iron in the ferrous state Porphyrins may be thought of as derivatives of *porphin*, which consists of four pyrrole rings Porphyrins are compounds in which the hydrogens of the pyrrole rings of porphin are replaced by various side chains In the porphyrin of the heme of hemoglobin three different types of side chains are present on the porphin nucleus—four methyl, two vinyl, and two propionic acid Porphyrins with this particular complement of side chains are called *protoporphyrins* There are fifteen possible isomeric protoporphyrins The structure of the naturally occurring isomer was determined by Hans Fischer and his pupils as identical with that which they arbitrarily assigned the number 9 It is, therefore, known as *protoporphyrin IX* (formula II)

Various other derivatives of porphin are known, of which one is chlorophyll (see page 405) *Coproporphyrin III* (formula III) is a normal constituent of human feces and, in very low concentration, of urine *Uroporphyrin I* (formula IV) occurs in the urine in the disease congenital porphyria (see page 664) The vinyl groups present in hemoglobin seem necessary for attachment of iron (5) The iron is actually bound by the four nitrogens, with the possibility of binding two more atom groups above and below the planar surface Each heme is attached to globin at three places two ionized propionic acid chains are held by two basic groups of protein, and the third link is by iron to a specific group on protein, possibly imidazole nitrogen This is the fifth co ordination link which may keep the iron ferrous The sixth co ordination link may be reversibly held by oxygen The methyl side chains remaining fulfill no obvious function and may serve merely to protect the porphin ring system from undesirable side reactions

Structure of Hemoglobin

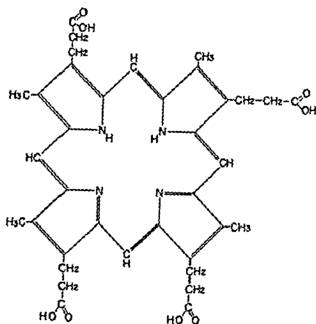
A large number of different nomenclatures for hemoglobin and its derivatives has been proposed, here we have chosen to follow the nomenclature of Lemberg and Legge (11) The iron in hemoglobin even when the hemoglobin is in combination with oxygen, is in the ferrous state, and measurement of magnetic susceptibility shows that the compound is diamagnetic and that the iron bonds are therefore covalent The oxygen molecule normally contains two unpaired electrons and it is evident that it has



II

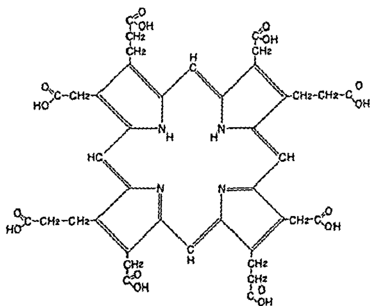
changed profoundly by combining with hemoglobin and the system resonates between the two structures A and B, where the electrons in the third orbitals of iron are represented by dots (formula V) If the iron of hemoglobin is oxidized to the ferric state, we then have the compound called by Lemberg hemiglobin, and by others *methemoglobin* which no longer has the power of transporting oxygen

While man and other warm blooded animals make use of hemoglobin as the oxygen transporting compound, certain lower forms have red heme containing proteins of larger molecular weight possessing iron Other lower organisms possess a large protein molecule which contains copper but not porphyrins These compounds are called *hemocyanins* Some snails have copper containing hemocyanin, others have iron containing compounds



C=O
OH
CH2
CH2
H
CH3
H3C
CH2–CH2–C=O
OH
NH
HN
HC
CH
N
N
H3C
CH3
H
CH2
CH2
HO
C=O
CH2
CH2
HO
C=O

III



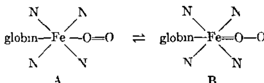
C=O
OH
CH2
CH2
H
C=O
OH
CH2
O=C–CH2
HO
CH2–CH2–C=O
OH
NH
HN
HC
CH
N
N
O=C–CH2–CH2
HO
CH2–C=O
OH
H
CH2
C=O
OH
CH2
CH2
C=O
OH

IV

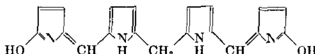
related to hemoglobin Some of the smaller snails have no detectable respiratory pigment

Hemoglobin Catabolism—Breakdown to Bile Pigments

The idea that bile pigment was derived from hemoglobin is quite old and was ascribed by Virchow to Breschet but the first clear evidence came from the observations of Virchow in 1857 We now know that bile pigment is formed in the organism by the oxidative rupture of the porphyrin ring of hemoglobin while it is still attached to iron and globin and before the red cell breaks up In this connection we must consider the life span of the red blood cell in the circulation Methods involving the use of radioactive tracers and blood grouping techniques have shown that the red



V Resonance structures of oxyhemoglobin



VI Bilirubin

cell survives in the circulation 100 to 140 days It has been supposed that as the cells circulate they gradually wear out and become progressively more fragile However it has been shown that values for hemolysis of samples of red cells are entirely compatible with the idea that it is purely a statistical matter whether or not a given red cell is hemolyzed

All of the bile pigments contain a structure of four pyrrole rings linked by methene bridges Of the large number of bile pigments and related substances which have been obtained and many of which occur in nature we restrict our discussion here to two bilirubin (formula VI) and biliverdin The terminations of the names of these pigments derive from Latin roots meaning red and green respectively

The structure of bilirubin includes two hydroxyl groups as shown It was observed by Paul Ehrlich in 1883 that this compound would couple with diazonium salts diazotized sulfanilic acid usually being used to give azo dyes This was developed by Hijmans van den Bergh as a standard method

of estimating bilirubin in serum Biliverdin contains two hydrogen atoms less than bilirubin, and has a green instead of a red color The sequence of reactions by which heme is converted into biliverdin and bilirubin is de scribed on page 463

THE ANEMIAS

The term *anemia* is generally used in clinical medicine to refer to a re duction below normal in the number of red corpuscles per cubic millimeter, the concentration of hemoglobin, the volume of packed red cells obtained by centrifugation Conversely, polycythemia designates a condition where the number of red cells is increased

There are a very large number of types of anemias, they are usually classified by laboratory examination of the blood If there is a proportion *ate decrease in the number of corpuscles, the quantity of the hemoglobin,* and the volumes of the packed red cells, indicating that the average con *tent of hemoglobin in the cell and the average size of the cell has* not been changed then this is called a *normocytic anemia* In some cases there is a greater decrease in the number of cells than in the volume of packed cells due to the fact that the majority of such red cells as are produced is larger than normal, this is called a *macrocytic anemia* More commonly, the re verse of this is found where the majority of the corpuscles is smaller than normal, this is called a *microcytic anemia* Wintrobe (22) may be consulted for a classification of anemias, their cause and their treatment The word anemia, as can be seen from its derivation, implies a lack of blood One of the most striking symptoms of anemic patients is pallor, indicating less hemoglobin underneath the skin

Macrocytic Anemias

Pernicious anemia has been conclusively demonstrated *by Minot and* Murphy to be due to deficiency of factors concerned in red cell maturation *It was shown not to be due to deficiency of iron Much later,* an anti pernicious anemia factor, cyanocobalamine (page 698), was isolated It is *not absorbed by patients affected with pernicious anemia* Normally, cyano-cobalamine is stored in the liver, and liver feeding was the first effective treatment of pernicious anemia, being later replaced by the use of con centrated liver extracts Other macrocytic anemias arise as a result of a deficiency of other substances necessary in the formation of red cells, such as folic acid

Microcytic Anemia

Microcytic anemia results from lack of iron Since the time of Hippocrates iron salts have been used for anemia by physicians *It has been stated that* the origin of this therapy dates back to sympathetic magic, because the

weak patient hoped to acquire the strength of steel by drinking water in which a sword had rusted Three centuries ago the use of iron in the form of filings which had been steeped in cold Rhenish wine was introduced by Sydenham into clinical medicine in treating a type of microcytic anemia chlorosis No doubt the acetic acid of the wine dissolved enough of the iron to benefit the patient In 1832, Pierre Blaud emphasized the specific value of iron in the treatment of chlorosis and described his now famous pills which contain ferrous carbonate

Post hemorrhagic anemia results from loss of blood, which of course involves a loss of iron It may be treated by blood transfusion or if it is not too severe, iron plus protein therapy may be used

The Normocytic Anemias

The anemia associated with the majority of chronic infections is sometimes called simple chronic anemia There is no very radical alteration from the normal red cell morphology The beginning is insidious and recovery is often very slow Myelophthisic anemia is associated with destructive processes in the bone marrow High protein diets have been effective in the treatment of simple chronic anemia The pathological changes leading to myelophthisic anemia, on the other hand, are frequently severe and irreversible so that no form of therapy has any prolonged effect

Hemolytic anemias include a large number of conditions which vary according to their cause and severity and have one feature in common which is excessive blood destruction The acute and subacute hemolytic anemias include anemias due to malaria, bacterial toxins, and chemical hemolytic agents Anemias due to blood destruction following mismatched blood transfusions or erythroblastosis fetalis otherwise known as hemolytic disease of the newborn or the action of cold agglutinins or the peculiar disease called paroxysmal hemoglobinuria, which is also dependent upon the action of cold, all fall in this class Paroxysmal hemoglobinuria is characterized by sudden passage in urine of hemoglobin following local or general exposure to the cold It was shown by Donath and Landsteiner in 1904 to be due to a sudden hemolysis of the blood by the action of a hemolytic agent contained in the patient's own blood which, however, was active only at low temperatures

Under the chronic hemolytic anemias we include familial or congenital hemolytic jaundice, sickle cell anemia (sicklemia), chronic hemolytic anemia with nocturnal hemoglobinuria, the so-called acquired hemolytic jaundice, and Cooley's anemia, otherwise known as thalassemia

Sicklemia

From the point of view of the chemistry of hemoglobin, anemias may be divided into two classes In the first class there is loss of hemoglobin

whether by destruction of red cells or some other mechanisms, but what remains is essentially normal hemoglobin All the anemias discussed previously fall into this class The second class includes sickle cell anemia, which is a hereditary form of a chronic anemia confined to Negroes or people with Negro ancestry In this disease the red cells on first examination appear normal, but if they are sealed up in a preparation without access to oxygen, they lose their customary shape and acquire the crescent shape which gives the name sickle cell anemia to the disease It has been shown by Pauling and co workers (15) that in this disease two forms of hemoglobin with different electrophoretic mobility are present, and it is likely that the difference between these hemoglobins is responsible for this disease This is one of the first cases in which the cause of a disease has been traced to a difference in the molecular form of one of the proteins present in a cell It has been suspected that Cooley's anemia, or Mediterranean anemia, is due to a somewhat similar cause, possibly even to the same gene acting in a single dose (see Chapter 9)

Blood Transfusion and Rh Factors

It was discovered by Landsteiner and his pupils about 1900 that all human blood could be classified into one of four groups, these are now designated as *O*, *A*, *B*, and *AB* Landsteiner, in his original papers, pointed out the possible importance of this for blood transfusion, but it was not until World War I that wide recognition of this importance prevailed, and from time to time transfusions of unmatched blood were attempted—sometimes successfully and sometimes with fatal results (1, 18, 19)

The phenomenon of isohemagglutination depends upon the fact that in the stroma of the red blood cells there are complex carbohydrates which we designate as A and B, called the blood group agglutinogens Some properties of the A blood group substance are shown in table 6 These may occur singly, together, or neither may occur, giving the four classical blood groups as shown in table 7 This, in itself, would not complicate transfusions However, in the liquid part of the blood there almost invariably occur complementary agglutinating substances, globulins in nature, according to a rule which may be formulated thus In your blood you will always find agglutinins for the people unlike yourself—never for your own blood When red blood cells containing agglutinogen A, for example, are mixed with plasma containing anti A agglutinin, the red cells stick together, or agglutinate This leads to such dangerous consequences as blockage of small blood vessels, hemolysis, and release of histamine Shock and death often result

It will also be seen that if group B blood is transfused into a person of group A, the anti B agglutinin in the plasma of the group A individual will

react with the introduced red cells, agglutinating them, and if complement is present, as it usually is, causing them to dissolve and producing a serious or fatal reaction Transfusion reactions are not always fatal, but are sufficiently dangerous to make it absolutely essential to determine the blood groups of recipient and donor before attempting a transfusion The agglutinin in the blood of the donor may also have bad effects but is not so important since it is considerably diluted—not more than 0 5 liters of blood being usually administered—to restore a circulating volume of some 7 to 8 liters Also, dissolved blood group antigens corresponding to the patient's own agglutinogens are found in his various tissues and body

TABLE 6

Chemical properties of human blood group A substance (10)

GROUP AND SUBGROUP	ASH AS Na	N	REDUCING SUGARS AS GLUCOSE	GLUCOSAMINE	ACETYL	OPTICAL ROTATION [α] 5893
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	
A_1	0 3	5 5	61	30	10 1	−13°
A_2	[1 3]	5 6	56	30	[7 2]	−27 5°

TABLE 7

Classification of blood groups

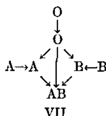
SUBSTANCE IN CELLS	AGGLUTININ IN SERUM	BLOOD GROUP
—	anti A and anti B	O
A	anti B	A
B	anti A	B
A + B	—	AB

fluids, and these help neutralize the introduced agglutinins These facts permit us to set up the possibilities of transfusion shown in formula VII

Nevertheless, it is better not to follow this scheme unless it is absolutely impossible to obtain a person of exactly the same blood group as the patient Persons of group O are often called universal donors, and it is true their blood has often been used in transfusions into people of other groups However, if their anti A and anti B agglutinins have a high titer and are unusually avid, they may react with the red blood cells of the recipient Many cases are on record in which this has occurred In a case observed by one of the authors (13), 25 per cent of the red blood cells of the recipient had been destroyed by the introduced agglutinins from the donor It is obvious that in such cases no good, but actual harm is done by the transfusion During World War II, when fresh refrigerated blood was being

flown from this country to the theaters of operation, only group O blood was sent, to avoid the necessity of doing a grouping before performing the transfusion. As a routine precaution, before blood was flown across, a 1:80 dilution of the plasma was tested against known sensitive A and B red blood corpuscles. This was a *purely arbitrary procedure, but it was* found by experience that it excluded the so called dangerous universal donors—that is, persons of group O who have an unusually high titer of anti A and anti B agglutinins. Their blood could still be used for conversion to dried plasma.

Antigenic differences among normal human bloods are not confined to the OAB systems of antigens, although these are by far the most important for transfusions. In 1927 Landsteiner *and* Levine *discovered three* new factors, M, N, and P. Agglutinins for these agglutinogens are not usually found in human plasma, however, and M and N are detected by the reactions of the absorbed sera of rabbits which have been injected with washed red



VII

cells of these types. It might have been supposed that it would be necessary to pay close attention to the differences in M, N, and P for transfusion as well as A and B, and this was in fact suggested. Actually, blood of type N could be transfused into patients of type M repeatedly without causing the formation of dangerous antibodies. The same apparently was true for P and other combinations of these types.

The M antigen is found to exist in the anthropoids as *indeed do the* O, A and B but in the case of M there seems to be a marked difference because some absorbed sera which are very satisfactory for detecting M in human beings are quite unsatisfactory in the case of certain anthropoids. While studying this phenomenon, Landsteiner and Wiener in 1940 discovered a new blood factor by injecting the blood of Rhesus monkeys into *rabbits and* absorbing to remove the undesired agglutinins. From the *initial letters of the word* Rhesus *we get the symbol Rh.* Rh might have

especially those in which the recipient had been repeatedly [illegible] produced reactions involving the Rh factor. Soon after this Levine and

co-workers discovered that Rh was in fact responsible for most cases of a rare disease of infants called erythroblastosis fetalis or hemolytic disease of the newborn, which had been described by Diamond (3) previously

If a woman becomes pregnant with a fetus which carries the Rh positive factor and she herself is Rh negative, the antigen from the fetus may diffuse across the placenta either as whole cells or simply as dissolved antigen, and cause the production in her circulation of anti Rh antibodies (21) In this respect Rh seems to be a more potent antigen than the other minor M, N P, and so forth If a mother is so sensitized (although fortunately this happens very rarely—only about once in 400 births), the agglutinins which she forms may diffuse across the placenta into the circulation of the fetus and cause damage to the red cells of the fetus The disease which appears in the newborn is called erythroblastosis, since in stained preparations of the blood young red blood cells called erythroblasts are seen Other symptoms such as liver damage, edema, and jaundice are often involved In subsequent work it has been shown that the Rh factor is actually extremely complex (see page 392)

TABLE 8

Newer blood group systems

NAME	GENE SYMBOLS	DATE DISCOVERED
Lutheran	Lu^a Lu^b	1946
Kell	K, k	1946
Lewis	Le(a), Le(b)	1946
Duffy	Fy^a Fy^b	1950
Levay		1946
Gr		1946
Jobbins		1947
S	S *s*	1947

Since the discovery of the Rh factor a number of other blood group antigens have been discovered, mostly by British workers, by the technique of observing incompatibilities in people who seem to be otherwise of the same blood group These have only limited clinical importance, but are of interest to students of genetics and anthropology A brief summary is given in table 8

IDENTIFICATION OF BLOOD

Blood stains are at times found at the scene of violent crime and it is often important to be able to say positively that these stains are blood, since many other substances, such as dried chocolate syrup, resemble old blood stains closely It is often desired to detect small amounts of blood in

gastric samples, exudates, urine, and feces, all as evidence of internal bleeding

One of the traditional tests depends upon the liberation of the prosthetic group heme from hemoglobin The blood stain is extracted and the extract dried on a microscope slide, and then treated with glacial acetic acid and a little sodium chloride and heated until the acetic acid boils It is then cooled slowly Chlorohemin, which is generally referred to as *hemin*, crystallizes in chocolate colored, rhombic plates which are very characteristic Spectroscopic examination of blood to detect the characteristic absorption spectra of hemoglobin or its derivatives has also been used to identify extracts as containing blood Other methods of detecting blood which are not specific depend on the fact that hemoglobin acts as a peroxidase One of the tests is the guaiac test The extract is treated with a few drops of an alcoholic solution of gum guaiac and then with hydrogen peroxide If blood is present, a blue color due to the oxidation of the guaiac to guaiaconic acid is obtained

Another somewhat more sensitive test is the benzidine test The suspected solution is treated with a saturated solution of benzidine in glacial acetic acid Hydrogen peroxide is added, and in the presence of blood, a brilliant greenish-blue or blue oxidation product of benzidine forms This test will detect blood in a dilution of about 1 in 1,000,000

After blood has been detected, it is necessary in medico-legal cases to determine whether or not it is human blood, for the presence of animal blood does not indicate murder This may be done by the precipitin reaction (1)

REFERENCES

1 Boyd, W C *Fundamentals of Immunology* Interscience Publishers, New York, 1947

2 Cohn, E H and Edsall, J T *Proteins, Amino Acids, and Peptides* Reinhold Publishing Corp, New York, 1943

3 Diamond, L K, *et al* Erythroblastosis fetalis and its association with universal edema of fetus, icterus gravis neonatorum, and anemia of newborn J Pediat, 1: 269-309, 1932

4 Edsall, J T The plasma proteins and their fractionation Advances Protein Chem, 3: 384-479, 1947

5 Granick, S Iron and porphyrin metabolism in relation to the red blood cell Ann New York Acad Sc, 48: 657-681, 1947

6 Gutman, A B The plasma proteins in disease Advances Protein Chem, 4: 155-250, 1948

7 Hald, P M The determination of the bases of serum and whole blood J Biol Chem, 103: 471 492, 1933 The importance of removing phosphorus in the determination of serum sodium *Ibid*, 130 133, 1939 Notes on the determination and distribution of sodium and potassium in cells and serum of normal human blood *Ibid*, 163: 429, 1946

8 HOWE P E The use of sodium sulfate as the globulin precipitant in the determination of proteins in blood J Biol Chem 49 93 107 1921

9 JELLINEK E M AND LOONEY J M Statistics of some biochemical variables on healthy men in the age range of twenty to forty five years J Biol Chem 128 621–630 1939

10 KABAT E A *et al* Immunochemical studies on blood groups IV J Exper Med 85 685–699 1947

11 LEMBERG R AND LEGGE J W *Hematin Compounds and Bile Pigments* Interscience Publishers New York 1949

12 MACFARLANE R G Discussion on the factors concerned in blood coagulation and their clinical significance Proc Roy Soc Med 38 395–405 1945

13 MAI IEL S AND BOYD W C A transfusion reaction due to a dangerous universal donor J A M A 129 344 1945

14 ONCLEY J L *et al* Physicochemical characteristics of certain of the proteins of normal human plasma J Phys and Colloid Chem 51 184 198 1947

15 PAULING L *et al* Sickle cell anemia a molecular disease Science 110 543–548 1949

16 PILLEMER L AND HUTCHINSON M C The determination of the albumin and globulin contents of human serum by methanol precipitation J Biol Chem 158 299–301 1945

17 QUICK A J *The Hemorrhagic Diseases and the Physiology of Hemostasis* pp 64 79 Charles C Thomas Springfield Ill 1942

18 SCHIFF F AND BOYD W C *Blood Grouping Technic* Interscience Publishers New York 1942

18a SEEGERS W H AND SHARP E A *Hemostatic Agents* Charles C Thomas Springfield Ill 1948

19 WIENER A S *Blood Groups and Transfusion* 3rd edit Charles C Thomas Springfield Ill 1943

20 WIENER A S AND PETERS H R Hemolytic reactions following transfusions of blood of homologous group with three cases in which same agglutinogen was responsible Ann Int Med 13 2306–2322 1940

21 WIENER A S AND WEXLER I B Transfusion therapy of acute hemolytic anemia of newborn Am J Clin Path 13 393 401 1943

22 WINTROBE M M *Clinical Hematology* 2nd edit Lea & Febiger Philadelphia 1946

23 WYMAN J JR Studies on the dielectric constant of protein solutions I Zein J Biol Chem 90 443–476 1931

PART II

Control

CHAPTER 5

Enzymes

In Section I we have discussed the chemical nature of the materials composing the human body This means thus far that the body has been considered as a static phenomenon In reality it is a system in which the various components are in a state of rapid flux and are related to and dependent upon one another in complex fashion Nothing in the body remains static, not even the inorganic constituents of the bones and teeth and the remainder of the book will therefore deal with the human body as a *dynamic* phenomenon

Before the actual interrelationships of body constituents can be taken up it is necessary that some time be spent on those internal factors which control or supervise the chemical reactions within the human body The processes which lead to birth, growth, and maturation to the conversion of food into living tissue and energy, are obviously not random or unorganized If they were they would continue unaltered by death and it is the very essence of life and death that they do not Even a relatively slight change in the nature of one of the many thousands of chemical reactions proceeding in the living body may result in serious illness or death Consider the extraordinarily small quantity of cyanide which is required to bring the entire mechanism of life to an abrupt halt Conversely, all maladjustments of the human organism involve—originally or eventually—the non function or malfunction of one or more of these reaction supervising factors The nature of these factors and their normal functioning becomes therefore a matter of vital interest

What are these factors? We will begin by simply supplying them with a name *enzymes* (derived from the Greek words meaning 'in yeast' because it was in yeast that enzymatic reactions were first systematically studied) Enzymes were recognized and their catalytic activity extensively studied long before any reliable data were available as to their chemical natures

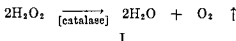
THE NATURE OF ENZYMES

Enzymes as Catalysts

An *ideal catalyst* is a substance which when present in small quantities will alter the rate of a chemical reaction without itself being altered in the

process An ideal catalyst would be expected, therefore, to exert its rat changing effect over indefinite periods of time if sufficient quantities of th reagents concerned are present Actually, no real catalyst is ideal, and a are, in the course of time as the catalyzed reaction proceeds, rendered ine or "poisoned"

Well known inorganic catalysts affecting many reactions include man metals such as platinum, palladium, or nickel, and such compounds a vanadium pentoxide and copper chromite Water itself is perhaps the mos versatile and important inorganic catalyst known, since a surprisingly wid array of reactions exist (the chemical union of hydrogen and oxygen t form water being the best known) in which the presence or absence of trace of water effects a tremendous change in rate Within the body ther exist catalysts (enzymes) which, by contrast, are complex organic com pounds However, as far as the basic principles of catalysis are concerned no distinction can be made between catalysts derived from living or non living sources Thermodynamically, the enzyme is no more esoteric thar platinum or water



$$2H_2O_2 \xrightarrow[\text{[catalase]}]{} 2H_2O + O_2 \uparrow$$

I

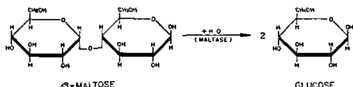
Let us consider several of the properties that all catalysts of whatever nature share A catalyst has already been defined as a substance which alters the rate of a reaction It is important to realize how limiting such a definition is It says nothing about affecting the nature of a reaction, its direction, or its extent, it affects only the rate The implication is that any reaction taking place under the influence of an enzyme (or any other catalyst) would also take place, albeit at a different rate, in the absence of an enzyme (or any other catalyst) This is indeed, true As a case in point let us consider hydrogen peroxide

When hydrogen peroxide solution is placed upon an open cut, there is a rapid effervescence which can be determined chemically to be due to the decomposition of the compound into water and oxygen (formula I) This decomposition of hydrogen peroxide is extraordinarily rapid, and takes place with similar rapidity in the presence of almost any type of tissue This is attributed to the widely distributed presence of an enzyme catalyz ing the reaction This enzyme has been given the name *catalase* This cata

of the enzyme, catalase, is not to change this reaction, but merely accelerate it many thousand fold

The case of hydrogen peroxide solution was chosen purposely because its decomposition proceeds in the absence of catalysis of any sort at a rate that is easily measurable In the case of many enzymatic processes, the corresponding reactions can not be shown to occur at all in the absence of the enzyme under comparable conditions Such would be the case, for instance, in the hydrolysis of maltose to glucose under the catalytic influence of the enzyme, maltase (formula II) Here, maltose is a stable organic compound which in aqueous solution at neutral pH and body temperature will persist unchanged for extended periods Is this a case where the presence of an enzyme has done more than simply change the rate of reaction? Has it acted to initiate a reaction? The view generally held by enzyme chemists is that these questions must be answered in the negative Even here, maltose is supposed to hydrolyze spontaneously to form glucose, but at a rate too slow to be measured

The basis for such a conclusion obviously can not be chemical, but

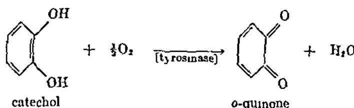


II

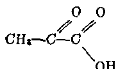
rather is thermodynamic in nature Thermodynamics is that branch of physics which deals with the interconversions of work and energy All substances can be considered to possess *free energy* (see Appendix III) The absolute value of free energy can not be calculated, but if the free energies of the various elements at certain defined standard states are set equal to zero, the relative free energies of compounds and of elements in states other than standard can be and, in many cases, have been calculated Thus, in the case of hydrogen peroxide decomposition the free energy of the hydrogen peroxide the water, and the oxygen (the temperature, pressure, and other environmental variables being known) has been calculated It turns out that the free energy of the hydrogen peroxide is greater than the combined free energies of the water and oxygen In other words, when hydrogen peroxide decomposes there is a net loss of free energy It is known from the laws of thermodynamics that *those reactions will occur spontaneously which involve a net loss of free energy* Thermodynamics, however, makes no predictions as to rates It states that the reaction will occur, given time, but whether a gram of hydrogen peroxide will decompose in one second or one year can not be predicted from the free energy change

alone, but depends on other factors, some of which will be discussed later in the chapter

Observe that it is the *net* loss of free energy that is significant In the body, a great variety of reactions take place in which compounds derived from foodstuffs are converted in the presence *of oxidizing enzymes to other* compounds of higher free energy content through the loss of two hydrogen atoms An example is the conversion of catechols to quinones by tyrosinase (formula *III*) It will be noted, however, that this is not the only chemical change which takes place In the process oxygen is reduced to water The loss in free energy in the conversion of oxygen to water is greater than the gain in the conversion of catechol to *o* quinone There is thus an over all or net, loss of free energy



III



IV Pyruvic acid

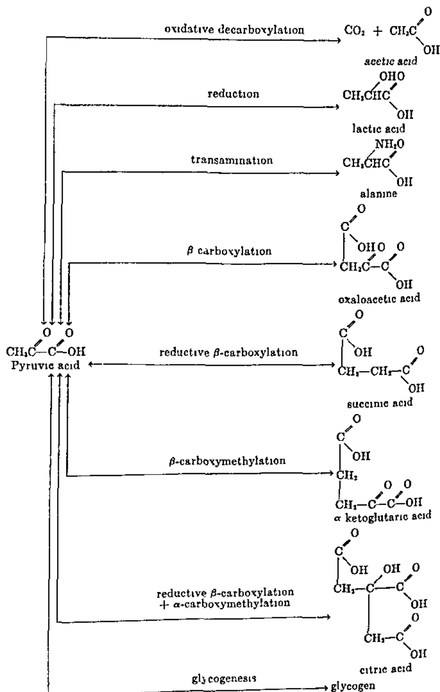
This same reasoning can be applied to living organisms in general The human body, in building itself up from a single fertilized egg to a com plicated mass of innumerable differentiated and complex cells, is performing *a process involving a vast gain in free energy* if growth and anabolism alone are considered However, the continual oxidation of foodstuffs in the body results in a loss of free energy greater than the gain involved in growth, so that the net change is a loss All foodstuffs are ultimately derived from plant life which utilizes solar energy to build up complex molecules from carbon dioxide, water, and inorganic salts Again the free energy loss involved in the absorption and utilization of solar radiation more than makes up for the free energy increase involved in the formation of carbohydrates *and proteins*

Enzymes, through their ability to accelerate thermodynamically possible reactions, are capable of determining the path of a reaction where several are possible Let us take the case of pyruvic acid (formula IV), an *impor*

tant intermediary in carbohydrate metabolism In the human organism pyruvic acid may undergo many chemical changes as shown in formula V In yeast cells, an additional change involving a simple decomposition can take place (formula VI) All these reactions can take place spontaneously, all involve loss of free energy at least in the first stages of the reaction Each reaction is catalyzed by a separate enzyme or group of enzymes However, all these changes do not take place in the body in any random catch as catch can way At a given time in a given tissue only certain of these reactions will take place perhaps, even only one of them One way of choosing among these multiple reactions is by controlling the chemical environment Thus in some of the reactions there must be a source of oxygen present, while in others there must be a hydrogen donator present, or a source of amino groups, and so on Granted that all such necessary substances are present the relative rates of the various reactions can be controlled by the presence or absence of specific enzymes Thus if only the enzyme transaminase were present, only the reaction it catalyzed would be accelerated in rate All the other reactions would still proceed, provided all necessary substances were present, but at their natural uncatalyzed rates The catalyzed transamination would so far outstrip the rest that all the pyruvic acid would be converted to alanine before significant quantities of the acid had reacted in any other way Thus the manner in which a reaction proceeds in the body depends both upon the environmental conditions and upon the nature of the enzyme or enzymes present

Until now, for simplicity's sake, we have treated free energy as an unalterable property of the material itself, whereas actually it varies with the concentration of the substance in the system The thermodynamic quantity which takes into account both the nature of the substance and its concentration is known as the *chemical potential* (see Appendix III) To be more precise than heretofore, it is the change in this chemical potential rather than in free energy itself which dictates the nature of chemical reactions that may take place in a given system

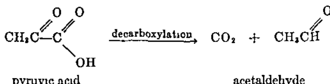
Let us consider the implications of this new concept with reference to a reaction such as the transamination of pyruvic acid and glutamic acid to form alanine and alpha ketoglutaric acid This reaction involves very little change in free energy If, however, we started with a mixture in which the concentrations of pyruvic acid and glutamic acid were considerably higher than those of alanine and alpha ketoglutaric acid, the chemical potential would be higher for the two former compounds The reaction would therefore proceed towards the formation of alanine Similarly, if the mixture contained considerably higher concentrations of alanine and alpha ketoglutaric acid than of the other two, their chemical potentials would be higher and the reaction would then spontaneously proceed in the


oxidative decarboxylation
CO₂ + CH₃COOH
acetic acid
reduction
CH₃CHOHCOOH
lactic acid
transamination
CH₃CHNH₂COOH
alanine
β carboxylation
oxaloacetic acid
CH₃COCOOH
Pyruvic acid
reductive β-carboxylation
succinic acid
β-carboxymethylation
α ketoglutaric acid
reductive β-carboxylation
+ α-carboxymethylation
citric acid
glycogenesis
glycogen

V

direction forming pyruvic acid and glutamic acid At certain sets of concentrations of the four compounds the sums of the chemical potential on each side of the equation are equal, and when this happens we have a state of equilibrium A reaction such as the one we have just discussed is known as a reversible reaction—that is, it can proceed in either direction according to the concentrations of the materials involved

Since a catalyst can not affect the free energy relationships of a reaction, *an enzyme can not alter the equilibrium point of a reaction* but can only change the rate at which equilibrium is reached from either direction Thus, the enzyme transaminase will catalyze the formation of alanine from pyruvic acid in the presence of glutamic acid, or with equal ease catalyze the formation of pyruvic acid from alanine in the presence of alpha keto glutaric acid In either case the same equilibrium point will be reached All reactions are in theory reversible, although in many cases the point of equilibrium is so far in one direction that to all practical purposes the *reaction may be considered as going to completion In reversing a reaction*



VI

physiologically it is therefore often necessary for the body to use an alternate route in which the equilibria concerned are more favorable Thus, in the hydrolysis of glucose-6 phosphate to glucose (see Chapter 12) equilibrium is heavily in favor of glucose 6 phosphate breakdown The formation of glucose 6 phosphate from glucose can not be, therefore, simply a reverse of the breakdown process catalyzed by the same enzyme, but must follow an entirely different route

Enzymes as Proteins

Until now we have discussed those properties that enzymes hold in common with all catalysts, it is now time to discuss those which differentiate them from other catalysts The differentiating property may be stated simply *Enzymes are proteins* Enzymes may therefore be completely defined as catalytic proteins The protein nature of enzymes was long unrecognized because of the very fact of their intense activity Extremely small concentrations of enzyme suffice to bring about very marked acceleration of the reactions they catalyze It was thus possible a quarter century or more ago to obtain purified enzyme extracts which, although very active yielded negative results to the protein tests of that day—even the most

sensitive That enzymes are proteins is indicated nevertheless by several lines of evidence

1 Enzymes exhibit properties entirely similar to those of proteins They are precipitated by protein precipitants, such as phosphotungstic acid or concentrated ammonium sulfate solutions, they will dissolve, as will proteins in water or in dilute salt solutions, they will not pass through a dialyzing membrane and they will lose their activity when exposed to any of the environmental factors known to denature proteins

2 When solutions of enzymes are allowed to stand at ordinary temperatures their activity declines with time This decline in activity is hastened by increasing the temperature If this decline is viewed as the result of some chemical action such a change in rate with increasing temperature is not unusual since the rate of all chemical reactions increases as the temperature is raised Whereas in the case of almost all ordinary chemical reactions the rate is only doubled or tripled for every ten-degree rise in temperature in the case of this decline in enzymatic activity with time the rate is increased many hundredfold for each ten degree rise in temperature There is only one other reaction known which shows a similar startlingly steep increase in rate with rise in temperature, and that is protein denaturation This seems strong evidence that enzymes are proteins and that their inactivation is due to the denaturing effects of, in this case heat

3 In 1926 Sumner isolated an enzyme in crystalline form for the first time in history (27)—an achievement for which he later received the Nobel Prize The enzyme was urease which catalyzes the hydrolysis of urea to carbon dioxide and ammonia Since then a number of other enzymes have been crystallized (23) In the case of every enzyme so crystallized examination showed it to be protein in nature

THE MULTIPLICITY OF ENZYMES

Terminology

The number of known enzymes is immense and as methods of investigation are refined it increases continuously It is useful, therefore to learn the systematic terminology for the individual enzymes The suffix *ase* is accepted as indicating an enzyme The rest of the name is usually chosen in one of the following ways

(1) From the name of the chief *substrate* (the substrate of an enzyme being the substance or substances affected by the reaction catalyzed) Thus urease is an enzyme which catalyzes the hydrolysis of urea

(2) From the name of the chemical process involved A *transphosphorylase* for instance, is an enzyme catalyzing the transfer of a phosphate group from one compound to another

These two systems of nomenclature can be combined as in ascorbic acid oxidase where both the substrate and the chemical process involved are named General groups of enzymes are named similarly A proteinase would be any one of many different enzymes which catalyze the hydrolysis of proteins An oxidase (or a reductase) is any of many different enzymes which catalyze an oxidation reduction reaction Closely related enzymes can be differentiated by including a reference to the nature of the environ ment in which they are active as in the case of acid phosphata e as opposed to alkaline phosphatase, or by naming the source from which the enzyme is derived as in the case of kidney phosphatase as opposed to intestinal phosphatase

Exceptions to these general rules occur among enzymes identified and studied during the infancy of enzymology before a systematic nomenclature had been developed, *e g*, catalase, where the substrate is hydrogen peroxide and the chemical process involves a dismutation Some of the digestive enzymes have names which even lack the almost universal *ase* suffix Pepsin and trypsin are the best known of these

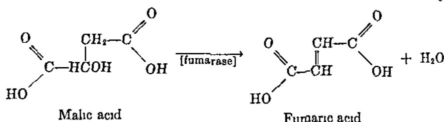
Pepsin and trypsin, as secreted by the gastric mucosa and pancreas, respectively, are inactive These inactive forms are termed *pepsinogen* and *trypsinogen* and differ from the active forms in possessing a polypeptide addendum which, apparently, shields certain reactive portions of the en zyme surface Such inactive precursors of enzymes are called *zymogens* Trypsinogen is activated by the enzyme, *enterokinase*, which occurs in the duodenal mucosa and whose action is to hydrolyze the shielding polypep tide off the enzyme surface Such activators of zymogens are called *kinases* Pepsinogen is not activated by a specific kinase, but rather through the proteolytic action of pepsin itself In other words, as soon as a molecule of pepsinogen spontaneously changes to form pepsin, the pepsin formed catalyzes the change of more pepsinogen into pepsin, which thus adds to the supply of catalyst and further increases the rate of reaction A system such as this where the product formed in a reaction is itself the catalyst of the reaction is an *autocatalytic system* Enzymes do not necessarily pass through a zymogen stage When, as in the case of pancreatic lipase, they are initially secreted in the active state, they are termed *preformed enzymes*

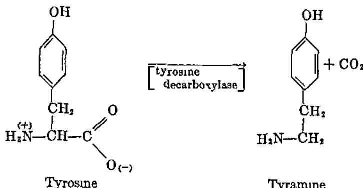
Classification

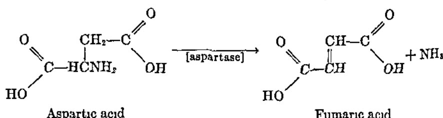
Baldwin (3) differentiates enzymes into two great groups and a third much smaller one These are (a) Splitting enzymes, (b) Transferring en zymes, and, (c) Isomerizing enzymes This is perhaps the broadest classi fication that can be made

Splitting enzymes. These catalyze reactions forming two or more mole cules out of one Three subgroups may be recognized First there are the

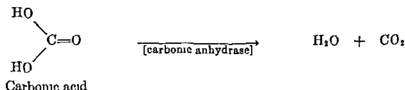
enzymes which catalyze simple decompositions—those where the molecule is split without the addition of extraneous atoms The reactions usually







VII



VIII

involve the separation of such simple groups as water, carbon dioxide, or ammonia Examples of such enzymes are fumarase, tyrosine decarboxylase, and aspartase, which catalyze reactions as shown in formula VII The en zyme *carbonic anhydrase, which catalyzes the decomposition of* carbonic acid to carbon dioxide and water, also belongs to this group (formula VIII)

There is next the subgroup of *hydrolyzing enzymes* or hydrolases which, as the name indicates, catalyze reactions that split compounds with the addition of the elements of water This subgroup, a large one, can be divided and subdivided on the basis of the nature of the bond split In general, it is sufficient at this point to state that one of four types of linkages is usually involved (a) —C—N—, (b) —C—O—, (c) —O—P—, and, (d) —N—P— As examples of enzymatic reactions involving each type, consider the hydrolytic cleavage of urea, acetylcholine, glyceryl phosphate, and phosphocreatine by *urease*, *cholinesterase*, *phosphatase* and *phosphamidase*, respectively (formula IX)

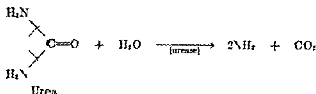
The third subgroup of the splitting enzymes is that which includes phosphorylyzing enzymes, *phosphorylases* These catalyze reactions that split compounds with the addition of the elements of phosphoric acid An example of such an enzyme is the phosphorylase which catalyzes the formation of the Cori ester (glucose 1 phosphate) from glycogen (formula X)

Transferring enzymes. These catalyze reactions in which a chemical group is transferred from one compound to another A large and very important subgroup of enzymes in this category are those catalyzing the transfer of two atoms of hydrogen from one molecule to another These are the oxidizing enzymes which will be discussed in detail later in the chapter An example of such an enzyme is the tyrosinase already referred to Enzymes which transfer groups other than hydrogen are less numerous Included are transaminases which have already been mentioned as well as transphosphorylases and transmethylases (formula XI) An unusual transphosphorylase is myokinase, which catalyzes a reaction in which adenosine diphosphate acts as both acceptor and donor of the phosphate group, forming adenylic acid and adenosine triphosphate (formula XII)

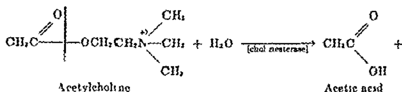
Isomerizing enzymes. These are a small group which can be looked upon as catalyzing the transfer of chemical groups from one position in a molecule to another position in the same molecule Examples of such enzymes are phosphotriose isomerase, and phosphoglyceromutase (formula XIII)

Specificity

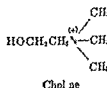
Implicit in the notion of enzyme multiplicity is that of enzyme specificity Since, except for the few enzymes whose crystalline forms can be characterized, enzymes can be differentiated only by the reactions they catalyze, it must follow that each enzyme is fairly specific in its action, otherwise differentiation and classification of enzymes would be meaningless That such specificity exists is easily demonstrated experimentally and is one of the most characteristic properties of enzymes In comparison, such non-enzymatic catalysts as platinum black, water, or light radiation



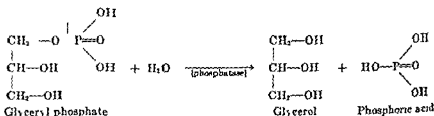
Urea
urease



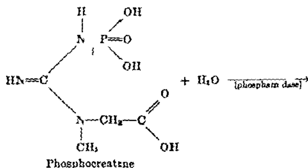
Acetylcholine
chol nesterase
Acetic acid



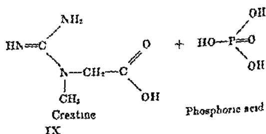
Choline



Glyceryl phosphate
phosphatase
Glycerol
Phosphoric acid



Phosphocreatine
phosphamidase

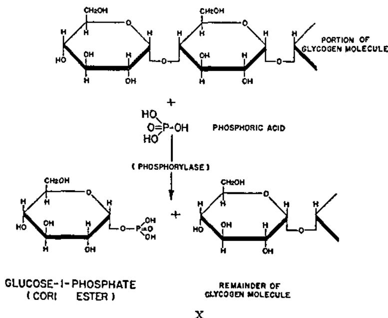


Creatine
Phosphoric acid

IX

are quite unspecific and catalyze all sorts of not necessarily closely related reactions

Absolute specificity. Many enzymes in fact, catalyze one reaction and one reaction only They act upon one and only one substrate Such enzymes show absolute specificity Examples are urease maltase, and succinic acid dehydrogenase Catalase is almost but not quite absolutely specific It has been shown to catalyze the decomposition of ethyl hydrogen peroxide to ethyl alcohol and oxygen (formula XIV)



X

Group specificity. Other enzymes display group specificity which indicates that provided a certain chemical grouping is present in the molecule, the nature of the remainder of the molecule is a matter of relative indifference This concept may be conveniently exemplified by a consideration of the group of enzymes known as *phosphatases*, which in the previous section have been listed as those hydrolases which split an —O—P— linkage The phosphatases can be divided into several subgroups *depending upon the* type of —O—P— linkage concerned Three of such subgroups are

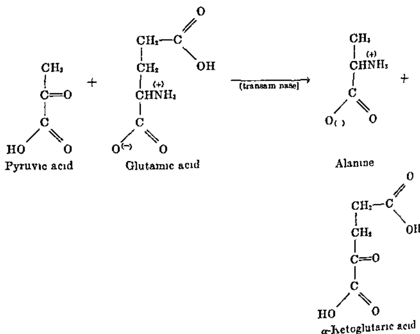
1 *Phosphomonoesterases*, the substrates for which must contain a singly esterified phosphoric acid group, e g , glyceryl phosphate (formula XV)

2 *Phosphodiesterases*, the substrates for which must contain a doubly esterified phosphoric acid group, e g , diglyceryl phosphate (formula XVI)

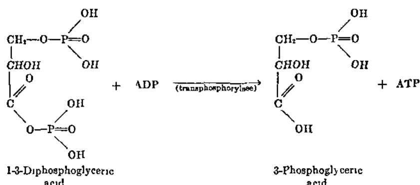
3 *Diphosphatases*, the substrates for which must contain two singly esterified phosphoric acid groups, e g , glyceryl diphosphate (formula XVII)

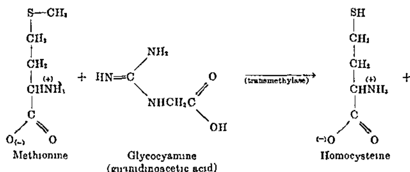
As far as the nature of the phosphate linkage is concerned the enzymes within each subgroup listed show absolute specificity As far as the nature of the organic radical with which the phosphoric acid is esterified, however, the enzymes are relatively *unspecific* Thus a *phosphomonoesterase* will hydrolyze phosphoric acid from such diverse compounds as glyceryl phosphate, phenyl phosphate, phenolphthalein phosphate, and adenylic acid and is therefore only group specific (formula XVIII) Even here, however, it should be noted that not all such compounds are hydrolyzed at equal rates, and the relative speed with which various mono esterified phosphoric acids are hydrolyzed varies with phosphomonoesterases isolated from various species

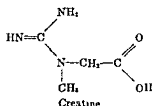
Enantiomorphic specificity. Enzymes catalyzing reactions involving such optically active substances as sugars or amino acids frequently act primarily or exclusively upon one of the enantiomorphs Arginase will act upon L arginine but not upon D arginine D amino acid oxidase, as its name indicates, will oxidize only D amino acids Furthermore, enzymes can form optically active compounds from inactive precursors Thus, lactic acid dehydrogenase, which catalyzes the interconversion of lactic acid and pyruvic



XI





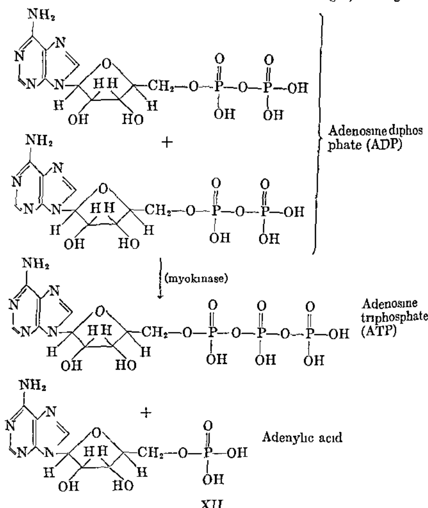


XI—*continued*

acid, will oxidize only L-lactic acid, and will form only L-lactic acid from the optically inactive pyruvic acid (formula XIX)

The protein hydrolyzing enzymes of the digestive juices present a special problem with regard to questions of specificity because of the complexity of their substrates From the fact that the human digestive system can hydrolyze most proteins one would suppose the enzymes involved to be relatively unspecific, or at most to display group specificity for the peptide

linkage However, the specificity involved is far more delicate than had been thought As examples we can take pepsin and trypsin Both will hydrolyze proteins or polypeptides of high molecular weight, forming as the



XII

end product not amino acids but rather peptides of moderate molecular weight The hydrolyzed products resulting from prolonged action of either enzyme will, however, yield readily to further hydrolysis by the other This would indicate that both pepsin and trypsin will hydrolyze only certain peptide linkages and that each will hydrolyze various linkages left untouched by the other By studies on artificial peptides of known composition Bergmann (4) showed that pepsin will hydrolyze a peptide bond

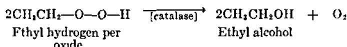
$CH_2—O—P(=O)(OH)_2$ / $C=O$ / CH_2OH → (phosphotriose isomerase) → $CH_2—O—P(=O)(OH)_2$ / $CHOH$ / CHO

Phosphodihydroxyacetone — 3 Phosphoglyceraldehyde

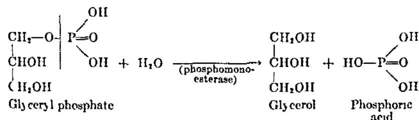
$CH_2—O—P(=O)(OH)_2$ / $CHOH$ / $COOH$ → (phosphoglyceromutase) → $CH_2—OH$ / $CH—O—P(=O)(OH)_2$ / $COOH$

3 Phosphoglyceric acid — 2 Phosphoglyceric acid

XIII

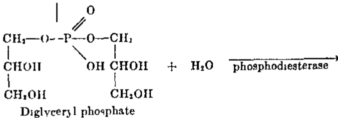


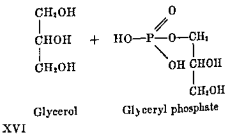
XIV



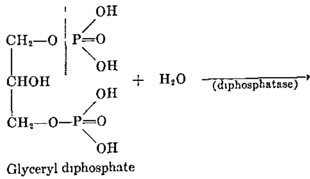
XV

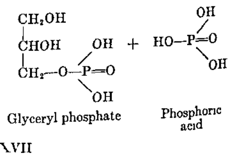
which is on the amino side of a residue of phenylalanine or tyrosine, whereas trypsin will act upon the carboxyl side of a residue of lysine or arginine. Chymotrypsin will act upon the carboxyl side of a residue of phenylalanine or tyrosine (formula XX).





XVI





XVII

The peptide fragments remaining from the action of pepsin and trypsin are su … e tion … e from that end of the peptide chain containing a free carbox…

other end of the chain would analogously be attacked by an aminopeptidase There are specialized peptidases which will hydrolyze off only a particular residue, as for instance prolinase which will act only when proline is at the end of a chain In addition there are numerous dipeptidases which catalyze the final hydrolysis of dipeptides to amino acids Peptidases are sometimes distinguished from one another by the position of the peptide linkage catalytically attacked Thus pepsin, under whose influence non terminal peptide linkages can be directly hydrolyzed, is termed an *endo peptidase* whereas carboxypeptidase, which can catalyze the hydrolysis of a terminal amino acid residue is called an *exopeptidase*

It would thus seem characteristic of living tissue that groups of necessary chemical reactions are catalyzed by many specific enzymes rather than by a few unspecific ones The assembly line technique in industry has been anticipated (by the manner in which proteins are digested, for instance,) by several hundreds of millions of years in the living cell

THE STRUCTURE OF ENZYMES

Essential Protein Groupings

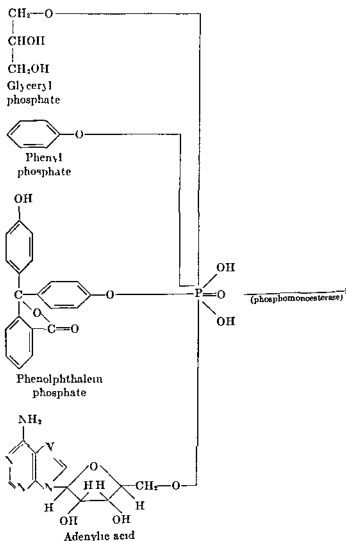
It is reasonable at this point to ask whether an enzyme is a typical protein or whether it possesses certain structural peculiarities that set it apart from those proteins which do not possess catalytic properties As far as we know now the former is the case In molecular weight, in amino acid content, in general structure, enzymes can not be differentiated as a class from other proteins Efforts have been directed at determining whether any particular portion of the enzyme molecule is responsible for its activity Thus pepsin acetylated to varying degrees has been tested for activity (23) It was found that acetylation first took place on the free amino groups of the three lysine residues of the pepsin molecule The activity of this triacetyl pepsin remained unchanged When further acetylation took place, activity declined by stages Since this further acetylation took place upon the phenolic side-chains of the tyrosine residues, there would appear to be a close relationship between the tyrosine of pepsin and the proteolytic activity of the protein molecule Similar results were obtained when pepsin was iodinated and the tyrosine residues converted to diiodotyrosine

In certain other enzymes, the thiol groups of cysteine or the amino groups of lysine have been found to be essential to catalytic activity Such experimental results must not be overemphasized Whereas a given grouping may have a direct connection with the actual catalytic process, it can not be considered as more important or more essential than any other portion of the enzyme In fact, any significant disruption of the enzyme structure, as in the case of all forms of denaturation, will decrease or erase enzyme

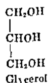
activity The capacity for catalytic activity is therefore obviously a function of the enzyme as a whole and not of any particular part alone

Prosthetic Groups

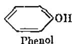
Not all enzymes are simple proteins Cytochrome oxidase and catalase which are of universal occurrence in animal cells are heme proteins containing as a prosthetic group the same iron porphyrin compound which occurs



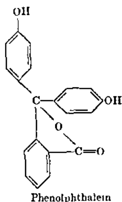
XVIII



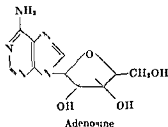
Glycerol



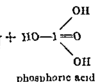
Phenol



Phenolphthalein

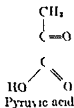


Adenosine

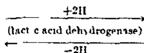


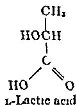
phosphoric acid

XVIII—*Continued*



Pyruvic acid





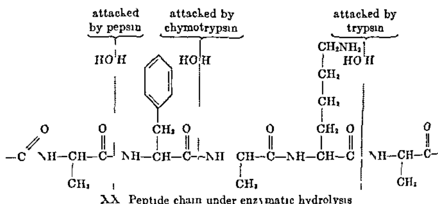
L-Lactic acid

XIX

in hemoglobin (see Chapter 4) Hemocyanin and certain plant oxidases contain copper closely associated with the protein molecule but the nature of the prosthetic group of which it may form a part is not yet known (7)

Activators

Many enzymes are without catalytic effect upon their substrates in the absence of definite amounts of certain ions, which do not appear to be so closely bound to the protein of the enzyme as to justify their consideration as parts of prosthetic groups The exact function of the ions is in many cases not known, and biochemists have been content to refer to them simply as activators In the case of the dipeptidases, however, Smith (20) has advanced evidence for the role of metallic activators as aiding in the forma



XX Peptide chain under enzymatic hydrolysis

tion of complexes between substrates and enzymes The complexes would be of the form of—


DIPEPTIDE—METALLIC ION—DIPEPTIDASE

with the metallic ion governing at least in part the specificity of the enzyme through the distribution of its co ordinate valences

The enzyme ion relationship is often not very specific In some cases it would seem that almost any bivalent metallic ion would do Thus yeast phosphatase can be activated by the bivalent ions of magnesium manganese, cobalt, nickel, and iron Enzymes are inactivated if the necessary

or if

is

lic

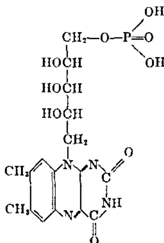
ions for their functioning

It should be mentioned that activators need not necessarily be metallic or even cationic in nature Salivary amylase requires chloride ion for any

activity whatever and the action of pancreatic lipase is accelerated in the presence of bile salts

Coenzymes

If an enzyme such as carboxylase is dialyzed, it is found to lose its activity If the inactive dialysate is added to the inactive dialyzed enzyme, activity is restored Dialysis removes a substance necessary to enzyme activity, a *coenzyme*, of relatively small molecular weight The coenzyme, unlike the protein itself, is thermostable, i e, is not inactivated by heating It is organic and not simply an inorganic ionic activator of the type we



XXI Flavin mononucleotide (FM) (coenzyme of cytochrome reductase)

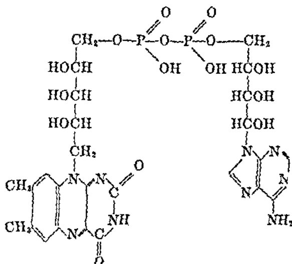
have already discussed, since if the dialysate is dried and ashed, activity after the re addition of water is lost In the case of carboxylase, the co enzyme has been identified as diphosphothiamine

Several other coenzymes have been isolated and their structure determined (formulas XXI–XXVI)

The manner in which at least some of these coenzymes function is known and will be discussed later in the section on oxidizing enzymes Certain of their characteristics, however, may be pointed out at this point Coenzymes do not generally display the sharp specificities of the protein portions of the enzyme (The protein portion is often referred to as the *apoenzyme*) As an example, DPN will act as coenzyme for alcohol dehydrogenase, lactic acid dehydrogenase, malic acid dehydrogenase, and so on In each case the apo enzyme itself is absolutely specific with respect to both the substrate and the coenzyme Enzymes which can make use of either of two coenzymes

are very rare It is thought that glucose dehydrogenase, *for instance, may* utilize either DPN or TPN as coenzymes To summarize, while a single apoenzyme can almost never make use of more than one coenzyme, a single coenzyme can often be used by several apoenzymes The situation is here similar to that whereby such proteins as hemoglobins of various species of animals, catalase, peroxidase, cytochrome c, and cytochrome oxidase (discussed later), differing widely in function, may all possess the same prosthetic group, heme

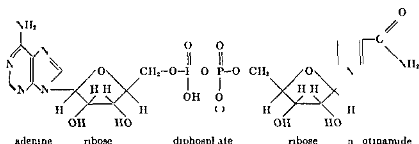
The coenzymes listed bear a close chemical relationship to several of the B vitamins It will be noticed that thiamine forms a portion of the



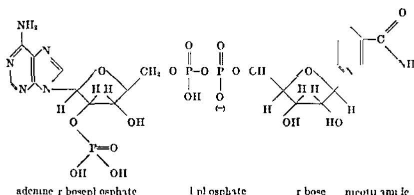
XXII Flavin adenine dinucleotide (FAD) (coenzyme of diaphorase)

diphosphothiamine molecule, riboflavin, a portion of FM and FAD, nicotinamide, a portion of DPN and TPN, and pyridoxal, a portion of pyridoxal phosphate (*formulas XXVII–XXX*) *In each case the individual B* vitamin may be looked upon as that portion of the coenzyme which can not be synthesized by the human being from simpler dietary components and which therefore must be supplied as such in the diet Naturally, since *coenzymes* are part of a catalytic system and therefore need be present only in trace amounts the vitamin content of a diet need not be very large to be adequate

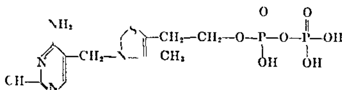
An inspection of the formulas presented for the vitamins and for the coenzymes of which they *form* part will show *that in each case* the vitamin must be phosphorylated before it can fulfill coenzymatic function Thiamine riboflavin, and pyridoxal are phosphorylated directly upon the al



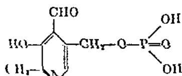
XXIII Diphosphopyridine nucleotide (coenzyme I or DPN) coenzyme of numerous dehydrogenases



XXIV Triphosphopyridine nucleotide (coenzyme II or TPN) coenzyme of numerous dehydrogenases

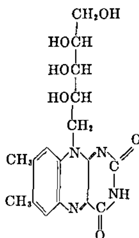


XXV Diphosphothiamine (coenzyme of carboxylase)

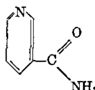


XXVI Pyridoxal phosphate (coenzyme of various transaminases and decarboxylases)

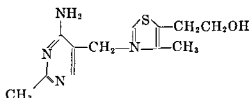
phatic hydroxyl contained in each molecule Nicotinamide is first condensed to a ribose residue which is then in turn phosphorylated In the case of DPN, TPN, and FAD, adenine is also part of the coenzyme molecule being linked to the phosphate by a second five carbon residue It should be emphasized that while this five carbon residue is D ribose in the case of DPN and TPN, it is the reduced derivative, the polyhydric alcohol D



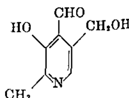
XXVII Riboflavin
(vitamin B_2)



XXVIII Nicotinamide
(niacinamide)



XXIX Thiamin
(vitamin B_1)



XXX Pyridoxal
(vitamin B_6)

ribitol in the case of both FAD and FM The number of phosphate mole

The term *nucleotide* is used for a molecule containing a
carbohydrate compound and the nitrogen of a ring system (see Chapter 7)

The role of other B vitamins in connection with coenzyme activity is also being investigated (29) Thus, pantothenic acid forms part of "Co enzyme A" (20) which is essential for various acetylation reactions in the

body, such as that catalyzed by an enzyme system in brain tissue involving the synthesis of acetylcholine from acetic acid choline and adenosine triphosphate, and biotin has been found essential in several physiological reactions The structures of the coenzymes of which these vitamins form part have not as yet been chemically determined, however It is reasonable to suppose that other trace dietary components either organic or inorganic, function via an enzyme system or systems

MEASUREMENT OF ENZYME ACTIVITY

Methods

The relative concentrations of the enzymes in slices, suspensions, or extracts of tissues may be estimated by measuring the rates at which the catalyzed reaction is proceeding In a reaction in which substance A is enzymatically converted to substance B, it is often possible to determine with great accuracy the concentration of either A or B at various times after the start of the reaction For example where catalase is catalyzing the decomposition of hydrogen peroxide, aliquots of the reaction mixture may be removed at given times, added quickly to a solution of molar sulfuric acid (which, by denaturing catalase protein, stops the enzyme reaction from proceeding further) and then titrated with potassium permanganate From the rate at which hydrogen peroxide disappears with time one can estimate the concentration of catalase Similarly when alkaline phosphatase catalyzes the hydrolysis of phenyl phosphate the rate at which phenol and inorganic phosphate are formed can be measured In some cases it is possible to determine directly the rate at which some component of the reaction system varies without its being necessary to remove aliquots This is possible in the hydrolysis of phenolphthalein phosphate in the presence of alkaline phosphatase As free phenolphthalein is formed under the alkaline conditions of the experiment, it of course turns red If the reaction is conducted in a cell of some colorimetric instrument, the increase in intensity of color with time can be measured continuously

Where the enzymatically catalyzed reaction involves either the utilization or the formation of a gas measurements can be made with great ease and refinement by *manometric methods* In these methods the enzyme reaction is allowed to proceed in a vessel which is attached to a manometer, a device which measures changes in gas pressure by the rise and fall of a column of liquid Changes in gas volume as small as 5 cubic millimeters (0 005 ml) can be easily detected Manometric methods are particularly adapted to the study of respiration of tissue slices since this involves the interchange of oxygen and carbon dioxide It can also be used to measure the rate of oxygen formation during the decomposition of hydrogen peroxide in the presence of catalase and the rate of carbon dioxide formation

resulting from the decomposition of various amino acids by the decarboxylases present in bacterial suspension. Many variations have been rung on this theme, many refinements and adaptations evolved to meet specific problems, so that manometry has become a large and highly specialized branch of enzymatic analysis. For further information concerning it, the student is referred to the monograph by Umbreit *et al.* (28).

The concentration of enzyme determined by rate measurements is not usually expressed absolutely as weight of enzyme per volume of solution. It is almost impossible to determine activity in terms of weight of enzyme unless one first purifies and crystallizes it and then determines the activity of a known weight of the crystals. This would also involve the assumption that the enzyme crystals were unchanged in efficiency as compared with the enzyme originally present in the tissues. It is much more convenient to express enzyme concentration relatively in terms of the reaction rates themselves. Concentrations are then expressed simply as *units*. Thus a unit of catecholase has been defined as that concentration of enzyme which under certain specified environmental conditions will oxidize catechol to quinone at a rate involving the uptake of ten cubic millimeters of oxygen per minute. The number of cubic millimeters (or microliters) taken up per milligram of tissue or specified tissue component per hour is usually symbolized as Q_{O_2}.

It is not necessary to use well defined chemical reactions in measuring enzyme activity. Amylase activity, for instance, can be measured in terms of the time required to hydrolyze a given weight of starch under standardized conditions to the point where it will no longer give the characteristic blue color with iodine or with potassium triiodide. In many cases the chemical reaction involved in the enzyme action may be ignored completely and a very accurate measure of activity obtained by observing changes in a selected physical property. Invertase, as an example, will hydrolyze sucrose to glucose and fructose. The optical rotation of sucrose is clockwise, whereas that of the equimolar mixture of glucose and fructose (due to the intense levo rotatory property of fructose) is counterclockwise. If the enzymatic hydrolysis is conducted in a polarimeter, the activity of invertase (hence its relative concentration) can be measured by the rate of change of optical rotation of the solution. Invertase, in fact, obtains its name from the fact that it changes or "inverts" the sign of the optical rotation. Nucleases, through their action in depolymerizing the long asymmetric molecules of nucleic acids (see Chapter 7), cause solutions of those substances to become less viscous and to lose their anisotropic properties. The rate of decline of either viscosity or anisotropy can thus be used as a measure of nuclease activity.

Two precautions must be emphasized and re-emphasized in the use of

the indirect method of determining enzyme concentration by enzyme activity. In the first place, *measurements of enzyme activity must always be conducted under rigidly controlled environmental conditions*, or they are meaningless. The nature and importance of some of the environmental conditions concerned will be discussed immediately below. Secondly, it often happens that different workers investigating a particular enzyme will define varying units. This may happen because they will

1. Use a different chemical reaction as a measure of enzyme activity—one may determine phosphatase activity by its catalytic effect upon phenyl phosphate and another by its effect upon glyceryl phosphate,
2. Use different aspects of the same reaction—one may determine phosphatase activity by using phenyl phosphate and measuring the free phosphate formed and another will measure the free phenol formed,
3. Using the same aspect of the same reaction but under varying environmental conditions—one may determine phosphatase activity at 25°C. and another at 37°C.

In doing any work on enzymes it is always advisable to make use of units already established in the literature, rather than to invent new ones however convenient the latter alternative might seem. Again, in comparing one set of data in the literature with another, or either with your own, particular attention must always be paid to the exact manner in which the units of enzyme activity were determined.

Environmental Effects

Nature of the enzyme. Since enzymes are proteins it should not be surprising, in view of the complexities of protein structure, that different samples of what would appear to be the "same" enzyme would vary in their properties. Many enzymes not only vary from species to species but also from tissue to tissue within a given animal. In many cases the properties of a given enzyme may depend upon the particular method used in isolating it. It is therefore important to maintain the source and the method of isolation of enzymes as constant as possible.

Concentration of the enzyme. It is a fundamental assumption in the measurement of enzyme activity that the rate at which a catalyzed reaction proceeds is directly proportional to the concentration of enzyme. That this is generally so can be shown from the fact that doubling the amount of enzyme preparation used *usually* results in doubling the rate of the reaction. This is so, however, only as long as the enzyme concentration is comparatively small. As more and more enzyme is used, there comes a point where the reaction rate is no longer increased proportionately but begins to lag behind. The reason for this should not be difficult to see. As the concentration of enzyme increases, the reaction becomes so rapid that the supply

of reactants is insufficient to feed it at maximum In the case of the manometric measurement of ascorbic acid oxidase for instance, the use of excessive quantities of enzyme leads to a depletion of oxygen in the solution, since it is consumed faster than it can be replaced from the air filled portion of the vessel If the manometer is shaken more rapidly, or if an oxygen atmosphere replaces the air in the vessel, oxygen is dissolved at a greater rate and the concentration limit of enzyme that can be efficiently used is raised From this we see that in any experiment designed to measure enzyme concentration through reaction rates, it must be determined that *under the conditions of the experiment* a direct relationship between the two does indeed exist

Concentration of the substrate. The question of substrate concentration is merely the inverse of what has been discussed immediately above If we return to the case of ascorbic acid oxidase, it is obvious that if ascorbic acid is completely absent there will be no oxygen uptake regardless of the quantity of enzyme present As ascorbic acid is increased in concentration the reaction rate, as measured by oxygen uptake, rises For a short time it will rise in direct proportion with the substrate concentration and then it will begin to lag until a point is reached where further addition of ascorbic acid causes little or no increase in the rate of oxygen uptake Again this results from the fact that at small substrate concentrations, not enough exists in solution to keep the enzyme molecules functioning at maximum rate Once the substrate concentration is high enough to induce full enzyme activity, the addition of still more substrate will obviously not increase activity further The enzyme, in other words, is then working as quickly as it can and additional substrate must simply wait its turn It is desirable to measure enzyme activity in the region of this substrate plateau so that the decrease in substrate concentration during the course of the reaction does not affect the reaction rate Indeed it often happens that where still more substrate is added the reaction rate begins to decline again for reasons that are not as yet entirely clear In that case a definite *substrate optimum* exists, at which it is desirable to maintain substrate concentration during the course of activity measurements

Temperature. Chemical reaction rates are invariably affected by temperature change As has been stated before most reaction rates increase by a factor of two to three for every ten-degree rise in temperature This increase is symbolized as Q_{10} This rule applies to enzymatically catalyzed reactions as well Another factor peculiar to such reactions however, must also be considered As the temperature rises, the rate of enzyme inactivation through heat induced alterations in its protein structure increases by a factor of hundreds in that ten-degree interval Small variations in temperature which would not be expected to alter the rate of a non-enzymatic

reaction significantly may very easily render enzyme rate studies meaningless

pH Proteins in general are multivalent amphoteric acid base compounds and their properties are changed with change in pH This holds true specifically for enzyme activity If enzyme measurements are made in a series of buffered media under conditions in which only the pH is varied from experiment to experiment the rate of the catalyzed reaction will vary considerably In general the reaction will proceed most rapidly at a certain pH, and this is the *pH optimum* On either side of this pH optimum there is a decline in activity and often activity ceases at distances of as little as two pH units from the optimum Provided the departure from pH optimum is not too extreme or too prolonged activity can be fully restored by a return to optimum This would indicate that the decline in activity is due not to permanent changes in protein structure but to changes in the ionized state of the enzyme i e in the distribution of charge on the enzyme surface

Enzyme experiments are usually conducted in buffered solutions maintained at the optimum pH for that enzyme It should be noted that each different enzyme has its characteristic pH optimum and that known optima vary from a pH of 1 5 for pepsin to one of 9 7 for arginase Optima at either extreme of the pH range are rare however and the majority of enzymes possess optima nearer the neutral point The optimum may vary slightly with the substrate, the buffer used or with other factors in the reaction system Enzyme reactions will vary in rate with *ionic strength* (see page 11) so that in preparing a buffer, the nature and concentration of the salts used must be taken into account as well as the final pH

Enzyme Inhibition

The term enzyme inhibition is usually restricted to such loss of enzyme activity as results from the presence of small quantities of certain chemical substances Such *inhibitors* may be classified into three groups, according to the method whereby the inhibiting process takes place

Protein precipitants Of the three the least significant are those substances which attack the protein moiety of the enzyme specifically Trichloroacetic acid for instance which is an excellent protein precipitant is in small concentrations an enzyme inhibitor Other compounds which would act to precipitate proteins or to denature them in any way, such as by the oxidation or acetylation of essential groups would also by their presence inhibit enzyme activity Such inhibition is usually not very specific since obviously all enzymes would be vulnerable to any protein damaging agent

Activator inhibition. A more specific and more medically significant

of reactants is insufficient to feed it at maximum In the case of the manometric measurement of ascorbic acid oxidase, for instance, the use of excessive quantities of enzyme leads to a depletion of oxygen in the solution, since it is consumed faster than it can be replaced from the air filled portion of the vessel If the manometer is shaken more rapidly, or if an oxygen atmosphere replaces the air in the vessel, oxygen is dissolved at a greater rate and the concentration limit of enzyme that can be efficiently used is raised From this we see that in any experiment designed to measure enzyme concentration through reaction rates, it must be determined that *under the conditions of the experiment* a direct relationship between the two does indeed exist

Concentration of the substrate. The question of substrate concentration is merely the inverse of what has been discussed immediately above If *we return to the case of ascorbic acid oxidase, it is obvious that if* ascorbic acid is completely absent there will be no oxygen uptake regardless of the quantity of enzyme present As ascorbic acid is increased in concentration the reaction rate, as measured by oxygen uptake, rises For a short time it will rise in direct proportion with the substrate concentration and then it will begin to lag until a point is reached where further addition of ascorbic acid causes little or no increase in the rate of oxygen uptake Again this results from the fact that at small substrate concentrations, not enough exists in solution to keep the enzyme molecules functioning at maximum rate Once the substrate concentration is high enough to induce full enzyme activity, the addition of still more substrate will obviously not increase activity further The enzyme, in other words, is then working as quickly as it can and additional substrate must simply wait its turn It is desirable *to measure enzyme activity in the region of this substrate* plateau so that the decrease in substrate concentration during the course of the reaction does not affect the reaction rate Indeed, it often happens that where still more substrate is added the reaction rate begins to decline again for reasons that are not as yet entirely clear In that case a definite *substrate optimum* exists, at which it is desirable to maintain substrate concentration during the course of activity measurements

Temperature. Chemical reaction rates are invariably affected by temperature change As has been stated before, most reaction rates increase by a factor of two to three for every ten-degree rise in *temperature* This increase is symbolized as Q_{10} This rule applies to enzymatically catalyzed reactions as well Another factor peculiar to such reactions, however, must also be considered As the temperature rises the rate of enzyme inactivation through heat induced alterations in its protein structure increases by a factor of hundreds in that ten-degree interval Small variations in temperature which would not be expected to alter the rate of a non-enzymatic

reaction significantly may very easily render enzyme rate studies meaningless

pH. *Proteins in general are multivalent, amphoteric acid base compounds* and their properties are changed with change in pH This holds true specifically for enzyme activity If enzyme measurements are made in a series of buffered media under conditions in which only the pH is varied from experiment to experiment, the rate of the catalyzed reaction will vary considerably In general, the reaction will proceed most rapidly at a certain pH, and this is the *pH optimum* On either side of this pH optimum there is a decline in activity and often activity ceases at distances of as little as two pH units from the optimum Provided the departure from pH optimum is not too extreme or too prolonged, activity can be fully restored by a return to optimum This would indicate that the decline in activity is due not to permanent changes in protein structure but to changes in the ionized state of the enzyme, i e in the distribution of charge on the enzyme surface

Enzyme experiments are usually conducted in buffered solutions maintained at the optimum pH for that enzyme It should be noted that each different enzyme has its characteristic pH optimum and that known optima vary from a pH of 1 5 for pepsin to one of 9 7 for arginase Optima at either extreme of the pH range are rare, however, and the majority of enzymes possess optima nearer the neutral point The optimum may vary slightly with the substrate, the buffer used, or with other factors in the reaction system Enzyme reactions will vary in rate with *ionic strength* (see page 11) so that in preparing a buffer, the nature and concentration of the salts used must be taken into account as well as the final pH

Enzyme Inhibition

The term, enzyme inhibition, is usually restricted to such loss of enzyme activity as results from the presence of small quantities of certain chemical substances Such *inhibitors* may be classified into three groups, according to the method whereby the inhibiting process takes place

Protein precipitants *Of the three the least significant are those* substances which attack the protein moiety of the enzyme specifically Trichloroacetic acid, for instance, which is an excellent protein precipitant is in small concentrations an enzyme inhibitor Other compounds which would act to precipitate proteins or to denature them in any way, such as by the oxidation or acetylation of essential groups, would also by their presence inhibit enzyme activity Such inhibition is usually not very specific since obviously all enzymes would be vulnerable to any protein-damaging agent

Activator inhibition. A more specific and more medically significant

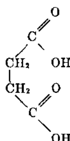
form of inhibition includes the effects of ions which are capable of forming complexes with metals whose presence is essential for enzyme activities Usually a *trace metal in order to function as an enzyme* activator must be unencumbered by chemical linkages with any groups other than those with which it is physiologically concerned, the enzyme and the substrate or substrates In the case of an iron enzyme a small concentration of cyanide ion would form a very stable ferro or ferricyanide complex which, by occupying the available co ordination linkages of iron in a more or less permanent fashion, makes it impossible for the iron further to fulfill its function Since the cellular respiratory system in man includes an iron enzyme, cytochrome oxidase, cyanide acts as a virulent poison

Competitive inhibition. Competitive inhibition is due to the lack of absolute specificity in complex formation on the part of the enzyme Succinic acid dehydrogenase, for instance, has already been described as possessing absolute specificity This is true insofar as it will catalyze the oxidation of succinic acid only However, if to a system containing the enzyme succinic acid (formula XXXIa), and oxygen, is added also malonic acid (formula XXXIb) the rate of oxygen uptake will decline If sufficient malonic acid is added, it will virtually cease This effect is not due to any *deleterious action of malonic acid upon the protein* It will be noticed, however, that malonic acid is very much like succinic acid—differing in fact only in that there is one less methylene group between the two carboxyls *It would seem then that in the formation of the intermediate* enzyme substrate complex, the enzyme is unable to distinguish completely between succinic acid and malonic acid In this respect the specificity is faulty The enzyme-malonate complex *can not be oxidized by oxygen and* in that respect the enzyme is specific However, the presence of the inert malonic acid upon the enzyme surface prevents those portions from combining with succinic acid In this manner enzyme activity is *inhibited* Since the formation of an enzyme substrate complex depends upon the rate at which the substrate under the influence of thermal agitation collides with the enzyme surface, which in turn depends upon the concentration of substrate, the extent to which malonic acid will inactivate succinic acid dehydrogenase depends upon the relative concentrations of succinic acid and malonic acid in the system If the … yme complex were as stable as the su… yme complex, … the molar concentrations of the tw… equal there … equal chance for either complex to be … enzyme acti… cut in half Actually, succinic acid … forms a … bond with succinic acid, for the u… ich its … ted, than … , interf… similarity … proper … ult it … lonic acid

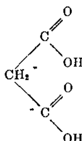
present in relatively large concentrations as compared with succinic acid in order to obtain a 50 per cent inhibition Similar behavior is observed in other systems subjected to competitive inhibition

Competition need not be between substrates alone Substances similar to but not identical with co enzymes or portions of co-enzymes will similarly compete for enzyme surface Much of chemotherapy is based upon this principle (see Chapter 20)

Still another form of competitive inhibition of medical interest is that existing among ionic activators As an example we may consider cases of beryllium poisoning following wounds received from broken fluorescent bulbs or from inhalation of the resulting dust Non healing granulomata in lungs or on skin are formed While the exact mechanism of the poisoning process is not yet known, it is considered significant that beryllium has



XXXIa Succinic acid



XXXIb Malonic acid

been found to inhibit competitively the functioning of alkaline phosphatase which requires magnesium as an activator Here the competition is between beryllium and magnesium two ions of identical charge and not too different size (1)

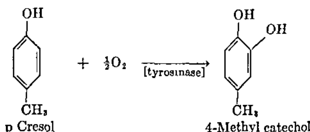
Enzyme Reaction Rates

Even where enzyme systems are carefully standardized with regard to temperature, pH and the other factors discussed above, the reaction rates we observe are seldom constant The velocities of reactions catalyzed by enzymes may decrease with time for any of the following reasons (a) the cumulative denaturing effect of temperature and gas liquid interface upon the enzyme (b) the decline in concentration of substrate as enzyme activity proceeds, and (c) the possible enzyme inhibiting nature of the products of the reaction *In vitro* the third item is the most difficult to deal with, since the first can be minimized by conducting enzyme reactions at low temperatures and the second by use of an excess of substrate In the case of many oxidizing enzymes, for instance, the oxygen used to oxidize the

substrates is itself reduced to hydrogen peroxide, a powerful protein denaturant Unless measures are taken to remove peroxide as quickly as it is formed, e g, by the addition of a small quantity of catalase, enzyme activity rapidly declines

It is therefore advisable to make measurements at intervals, graph the results, and choose that portion of the curve which is linear for calculations of enzyme activity Sometimes changes in enzyme activity are so rapid that no portion of the curve is linear When this occurs, mathematical devices may be used to determine the rate of *enzyme activity at zero time* (22)

Cases where velocity increases with time also occur Such periods of increasing reaction rates at the start of an enzyme reaction are known as *induction periods* An example of such a case is found in the action of the enzyme tyrosinase upon *p* cresol The first step of the oxidation is the



XXXII

conversion of *p* cresol to the corresponding catechol with oxygen being consumed (formula XXXII) When the reaction is followed manometrically it is found that, initially, oxygen uptake is very slow but increases until after several minutes it is proceeding at a linear rate When a small quantity of catechol is added to the system to begin with, however, oxygen uptake is linear from the very start Where catechol is excluded from the mixture, the enzyme can not perform its catalytic function until some has been formed by the non catalytic oxidation of the *p* cresol This would account for the initially slow but rapidly increasing rate of the reaction (5) Such induction periods can be detected and allowed for only by making periodic measurements during the course of the enzyme reaction

An absolute value for enzyme reaction rates can be obtained if a known weight of enzyme of known molecular weight is present in the system In such a case it is possible to calculate the number of molecules of substrate which react in a given time under standardized conditions for each molecule of enzyme present This quantity is known as the *turnover number* Catalase has an extraordinarily high turnover number One molecule of catalase will decompose five million molecules of hydrogen peroxide in

one minute at 0°C Turnover numbers in the thousands are much more common, and the value for Warburg's yellow enzyme is only fifty

BIOLOGICAL OXIDATIONS

Electron Chemistry

All of life, except that of viruses and anaerobic bacteria, is made possible by the fact that molecular oxygen can be made to combine with the carbon and hydrogen of foodstuffs to yield carbon dioxide, water, and *energy* It is this chemical fact that underlies the necessity for food and air, for eating and breathing Oxygen, on the one hand is a powerful oxidizing agent, capable under certain conditions of reacting with foodstuffs in order to yield comparatively large amounts of useful energy On the other hand it is sluggish in its actions, and will not spontaneously oxidize most foodstuffs Oxygen may thus be visualized as a powerful tool which remains quitely *in its case until needed and then, under the influence of the oxidizing* enzymes of the body, is capable of great feats To gain insight into this curious phenomenon and the life processes that depend upon it, a brief excursion into electron chemistry is necessary To begin with some knowledge of the electronic configuration of the biochemically significant elements is required

Electronic configuration of the elements. Beginning with the simplest elements, hydrogen possesses a single electron and helium, two They can be symbolized as H and He The higher elements all contain an inner shell of two electrons as in helium, and begin adding to an outer shell Since only the outermost shell of electrons in any given element is concerned in chemical reactions it is conventional to omit consideration of all but those and to symbolize lithium the third element, as Li —the two inner electrons being understood but not expressed The process continues an electron being added for each additional element, until a complete shell (containing eight electrons this time) is obtained in element number 10, neon, which would be expressed as Ne Once again, a new shell is begun, and element 11, sodium, is Na This new shell is completed with element 18, argon which is A

Electronic structures becomes considerably more complicated as atomic number increases All but the innermost electron shell are composed of four or more subshells, the electrons of which differ in energy content The energy spread among subshells becomes greater with each successive shell until, by the time the third shell is reached, overlapping with the next higher shell begins The result is, for instance, that potassium and calcium

represent atoms which have begun adding one and two electrons, respectively to the innermost subshell of the fourth electron shell, while the outermost subshells of the third electron shell are as yet unfilled The next ten atoms retain two electrons in the fourth shell and proceed to complete the electron content of the third shell Such overlapping grows continually worse with increasing atomic number, and results in the phenomenon of

TABLE 9

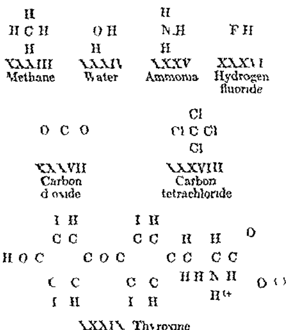
Electronic configuration of elements of biochemical interest

ELECTRON SHELLS	I	II	III	IV	V	VI	VII	VIII			0
1	1) H										2)
2	3)	4)	5)	6) C	7) N	8) O	9) F				10)
3	11) Na	12) Mg	13)	14)	15) P	16) S	17) Cl				18)
4	19) K	20) Ca	21)	22)	24)	25) Mn		26) Fe	27) Co	28)	
	29) Cu	30) Zn	31)	32)	33)	34)	35)				36)
5	37)	38)	39)	40)	41)	42)	43)	44)	45)	46)	
	47)	48)	49)	50)	51)	52)	53) I				54)
6	55) etc										

varying valence since the overlapping subshells are closely spaced (energically speaking) The heavier atoms may therefore avail themselves of only the outermost electrons or, on the other hand may use electrons from an inner subshell as well in forming valence bonds Thus iron and cobalt with two electrons in their outermost sub shells form many compounds in which they exhibit a valence of two Each however, may and frequently does utilize a third electron from an inner sub-shell to form compounds in which they possess a valence of three Similarly, copper with one electron in its outermost shell may draw two more from an inner shell while manganese with two electrons in its outermost shell may utilize as many as

five electrons from an inner shell These however are the only elements of biochemical significance (see table 9) which exhibit this phenomenon All others from hydrogen to iodine behave much more uniformly from an electronic standpoint

Electronic concept of valence In the formation of chemical compounds *from the elements* we can usually detect a tendency on the part of the elements concerned (particularly in the case of those of low atomic weight) to gain lose or share electrons in such a way as to end up with a



XXXIX Thyroxine

complete electron shell a state of maximum electronic stability The formation of simple compounds such as methane water, ammonia hydrogen fluoride carbon dioxide and carbon tetrachloride follows these electronic principles which thus account for the usual valence bond configurations The same principles apply with rare exceptions to organic compounds of greater complexity In the electronic formulas presented here (formulas XXXIII XXXIX) the electrons are pictured as little dots occupying particular places between two atoms Actually the picture is much more complicated and for the understanding of many problems in chemistry it is necessary to assume the electronic charges to be distributed among various portions of the molecule and to be spread about like jam rather

represent atoms which have begun adding one and two electrons, respectively, to the innermost subshell of the fourth electron shell, while the outermost subshells of the third electron shell are as yet unfilled The next ten atoms retain two electrons in the fourth shell and proceed to complete the electron content of the third shell Such overlapping grows continually worse with increasing atomic number, and results in the phenomenon of

TABLE 9

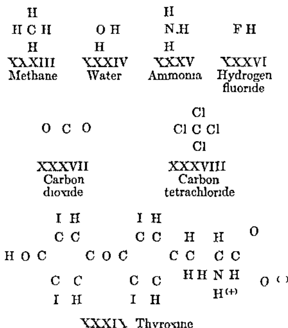
Electronic configuration of elements of biochemical interest

ELECTRON SHELLS	I	II	III	IV	V	VI	VII	VIII			0
1	1) H										2)
2	3)	4)	5)	6) C	7) N	8) O	9) F				10)
3	11) Na	12) Mg	13)	14)	15) P	16) S	17) Cl				18)
4	19) K	20) Ca	21)	22)	24)	25) Mn		26) Fe	27) Co	28)	
	29) Cu	30) Zn	31)	32)	33)	34)	35)				36)
5	37)	38)	39)	40)	41)	42)	43)	44)	45)	46)	
	47)	48)	49)	50)	51)	52)	53) I				54)
6	55) etc										

varying valence, since the overlapping subshells are closely spaced (energically speaking) The heavier atoms may therefore avail themselves of only the outermost electrons or, on the other hand, may use electrons from an inner subshell as well in forming valence bonds Thus iron and cobalt with two electrons in their outermost sub shells form many compounds in which they exhibit a valence of two Each, however, may and frequently does utilize a third electron from an inner sub shell to form compounds in which they possess a valence of three Similarly, copper with one electron in its outermost shell may draw two more from an inner shell, while manganese with two electrons in its outermost shell may utilize as many as

five electrons from an inner shell These however are the only elements of biochemical significance (see table 9) which exhibit this phenomenon All others from hydrogen to iodine behave much more uniformly from an electronic standpoint

Electronic concept of valence In the formation of chemical compounds from the elements we can usually detect a tendency on the part of the elements concerned (particularly in the case of those of low atomic weight) to gain lose or share electrons in such a way as to end up with a



XXXIX Thyroxine

complete electron shell a state of maximum electronic stability The formation of simple compounds such as methane water ammonia hydrogen fluoride carbon dioxide and carbon tetrachloride follows these electronic principles which thus account for the usual valence bond configurations The same principles apply with rare exceptions to organic compounds of greater complexity In the electronic formulas presented here (formulas XXXIII XXXIX) the electrons are pictured as little dots occupying particular places between two atoms Actually the picture is much more complicated and for the understanding of many problems in chemistry it is necessary to assume the electronic charges to be distributed among various portions of the molecule and to be spread about like jam rather

represent atoms which have begun adding one and two electrons, respectively to the innermost subshell of the fourth electron shell while the outermost subshells of the third electron shell are as yet unfilled The next ten atoms retain two electrons in the fourth shell and proceed to complete the electron content of the third shell Such overlapping grows continually worse with increasing atomic number, and results in the phenomenon of

TABLE 9

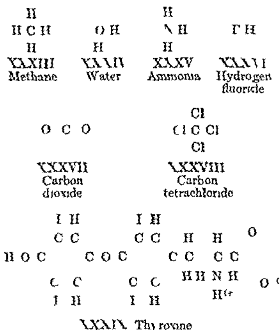
Electronic configuration of elements of biochemical interest

ELECTRON SHELLS	I	II	III	IV	V	VI	VII	VIII			0
1	1) H										2)
2	3)	4)	5)	6) C	7) N	8) O	9) F				10)
3	11) Na	12) Mg	13)	14)	15) P	16) S	17) Cl				18)
4	19) K	20) Ca	21)	2[illegible])	24)	25) Mn		26) Fe	[illegible]7) Co	[illegible]9)	
	29) Cu	30) Zn	31)	32)	33)	34)	35)				36)
5	37)	38)	39)	40)	41)	4[illegible])	43)	44)	45)	46)	
	47)	48)	49)	50)	51)	52)	53) I				54)
6	55) etc										

varying valence since the overlapping subshells are closely spaced (energetically speaking) The heavier atoms may therefore avail themselves of only the outermost electrons or on the other hand, may use electrons from an inner subshell as well in forming valence bonds Thus iron and cobalt with two electrons in their outermost sub shells form many compounds in which they exhibit a valence of two Each, however, may and frequently does utilize a third electron from an inner sub shell to form compounds in which they possess a valence of three Similarly, copper with one electron in its outermost shell may draw two more from an inner shell while manganese with two electrons in its outermost shell may utilize as many as

five electrons from an inner shell These however are the only elements of biochemical significance (see table 9) which exhibit this phenomenon All others from hydrogen to iodine behave much more uniformly from an electronic standpoint

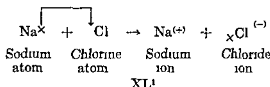
Electronic concept of valence In the formation of chemical compounds from the elements we can usually detect a tendency on the part of the elements concerned (particularly in the case of those of low atomic weight) to gain lose or share electrons in such a way as to end up with a



complete electron shell a state of maximum electronic stability The formation of simple compounds such as methane water, ammonia hydrogen fluoride carbon dioxid and carbon tetrachloride follows these electronic principles which thus account for the usual valence bond configurations The same principles apply with rare exceptions to organic compounds of greater complexity In the electronic formulas presented here (formulas XXXIII XXXIX) the electrons are pictured as little dots occupying particular places between two atoms Actually the picture is much more complicated and for the understanding of many problems in chemistry it is necessary to assume the electronic charges to be distributed among various portions of the molecule and to be spread about like jam rather

than placed like raisins Such concepts, usually referred to by the term *resonance*, are however beyond the scope of this book and for our purposes the simpler images here presented will be sufficient

Two major types of valence bonds may be formed, depending upon the electronic structure of the elements involved *Electrovalent bonds* are usually formed between elements of widely different electronic structure Sodium, for instance, having only one electron in its outer shell need only lose that one to gain a stable configuration, and therefore has a great tendency to lose it Chlorine, on the other hand, has seven electrons in its outer shell and has therefore a similarly great tendency to gain the single electron that it requires for maximum stability The reaction between them is one of complete transfer (formula XL) Sodium, with the loss of one electron is left with a net unit positive charge, while chlorine, having gained one, possesses a net unit negative charge They are no longer neutral elements but are now charged ions, which attract one another according to the laws of electrostatics Elements such as sodium which have a tendency to



XL[1]

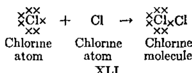
lose electrons are termed *electropositive*, while those like chlorine which have a tendency to gain electrons are termed *electronegative* Methods exist for measuring quantitatively the relative tendencies of elements to gain or lose electrons and scales of electronegativity and electropositivity have been prepared Of the elements of biochemical significance, sodium potassium, and calcium are among the electropositive elements, while oxygen nitrogen, and chlorine are electronegative Carbon and hydrogen are intermediate in character

Where elements are of similar electronic structure, *covalent bonds* are formed Here the electron transfer is incomplete, and the electron is more or less shared between the two and can be considered as forming part of the electron shell of each atom This is especially so in the case where the two atoms concerned are identical, as in chlorine gas Cl_2 (formula XLI), and the two electrons between the atoms are equally shared by both Since carbon and hydrogen are about equally electronegative (each having

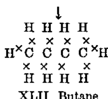
[1] In this and similar formulae the electrons of various atoms are distinguished as dots and crosses This is for ease of picturing the phenomena described and does *not* imply any difference in the nature of the electrons Actually the electrons of all elements are identical in nature

exactly half a complete shell of outer electrons) they also form compounds in which electrons are equally shared, as in butane (formula XLII) Where such equal sharing of electrons exists, compounds are termed *non polar*, since there is no net separation of electrical charge

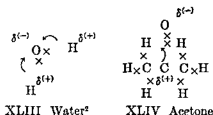
Where elements possess moderately different electronic structures a bond is formed which is intermediate in nature and is neither electrovalent nor yet entirely covalent In other words electrons are neither transferred



XLI



XLII Butane



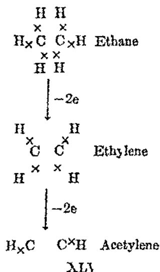
XLIII Water[2] XLIV Acetone

entirely nor are they shared equally They are shared unequally As examples we can consider the electronic structure of water (formula XLIII) and of acetone (formula XLIV) In each case the electrons binding oxygen and hydrogen or oxygen and carbon, are held more tightly by the oxygen since that element is more electronegative that either carbon or hydrogen The result is that there is an accumulation of some negative charge at the oxygen end of the molecule and of positive charge at the carbon or

[2] The symbols $\delta^{(-)}$ and $\delta^{(+)}$ are used to express fractional charges due to unequal electron sharing The charges so represented are usually less than unit charges which are symbolized in the usual manner as (+) or (−)

hydrogen end of the molecule Molecules in which separation of charge exists are termed *polar*

Water and ammonia are typical polar compounds, while the various hydrocarbons and such compounds as carbon tetrachloride are non polar Polar compounds tend to dissolve in polar solvents and non polar compounds in non polar solvents Organic compounds containing oxygen or nitrogen tend to be polar and if enough of these elements exist in the molecules they are water soluble For this reason the sugars and several amino acids are water soluble Where an organic compound contains a large hydrocarbon component as is the case in lipids and in such amino



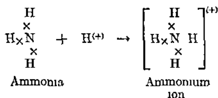
XLV

acids as phenylalanine it is sparingly soluble in water and relatively soluble in non polar liquids Non polar liquids are often referred to collectively as fat solvents

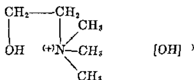
Double and triple bonds result from the sharing of four and six electrons respectively The formation of a double bond from a single bond or of a triple from a double involves the loss of electrons and is therefore an oxidative process This can be exemplified in the conversion of ethane to ethylene and then to acetylene (formula XLV)

Covalent bonds are not necessarily formed by the contribution of equal numbers of electrons by two different elements If we consider ammonia for instance we see that the nitrogen is surrounded by four pairs of electrons three of which are shared with hydrogens The fourth and last pair remains unshared It is possible for such an unshared pair of electrons to be used as a means of attaching a hydrogen ion as shown in formula XLVI

so that nitrogen would be surrounded by four hydrogens, and would possess a positive charge, becoming in fact, ammonium ion Such a bond in which one atom contributes both shared electrons is known as a *co ordinate* or *semi polar bond* Note that it is *not* a hydrogen *atom* which is attached to the nitrogen, since it would possess an electron of its own for which there would be no place in the octet The ammonium ion, since it is positively charged, can form an electrovalent link with a negatively charged ion such as chloride This fifth bond is *not* equivalent to the other four and should *not* be represented in similar fashion so as to indicate nitrogen to have a valence of five A number of compounds of biological interest contain nitrogen with four covalent links and a positive charge Examples are



XLVI



XLVII Choline

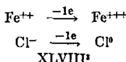
DPN, thiamine (the formulas for which have already been given) and choline (formula XLVII)

Electronegative elements, such as fluorine, oxygen, and nitrogen, accumulate a sufficiently large negative charge in compounds to possess considerable attraction for hydrogens which have accumulated positive charges through being attached to oxygen, nitrogen, or fluorine These are the *hydrogen bonds* so important with reference to protein structure

Electronic concept of oxidation-reduction. Oxidation has already been defined as a chemical process involving the loss of electrons Such a process is easy to visualize in simple ionic reactions as when ferrous ion is oxidized to ferric ion or chloride ion is oxidized to chlorine gas, in each case through the loss of one electron (formula XLVIII) The conversion of ferric ion to ferrous ion or chlorine gas to chloride ion would be the corresponding reductions, each reaction involving the gain of one electron It is not to be thought, however, that either oxidations or reductions can

exist independently Where ordinary chemical reactions are concerned, there are at no time measurable concentrations of free electrons present in solutions What does take place, therefore, is the coupling of an oxidation and reduction involving the transfer of electrons from the substance being oxidized to the substance being reduced An example of such a complete oxidation reduction reaction (or as it is sometimes called, *redox reaction*) can be obtained by combining the two partial reactions discussed immediately above Molecular chlorine will oxidize solutions of ferrous salts to ferric salts, itself being reduced to chloride ion (formula XLIX)

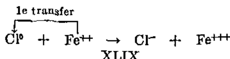
In the case of the non ionic reactions which are so important in biochemistry similar principles can be applied Thus when carbon burns in the presence of oxygen the process can be represented electronically as shown in formula L Since ions are not involved, there is no complete transfer of electrons However, it will be recalled that oxygen is far more electronegative than carbon, and has a stronger attraction for the shared



$$Fe^{++} \xrightarrow{-1e} Fe^{+++}$$

$$Cl^{-} \xrightarrow{-1e} Cl^{0}$$

XLVIII[*]



$$Cl^{0} + Fe^{++} \rightarrow Cl^{-} + Fe^{+++}$$

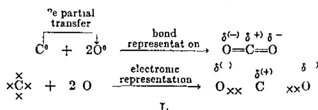
XLIX

electrons than has carbon The process may be considered on that account to be a partial transfer of electrons from carbon to oxygen, so that in the process carbon has been oxidized and oxygen reduced When atoms share electrons equally, as in the formation of a chlorine molecule from two chlorine atoms or the formation of any hydrocarbon from carbon and hydrogen, there is naturally neither oxidation nor reduction

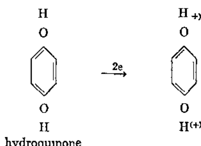
A special type of oxidation of great importance in biochemistry is that known as *dehydrogenation* Its electronic significance is exemplified by the oxidation of hydroquinone to *p* quinone (formula LI) In step 1, two electrons are lost, one from each pair shared by the hydrogens and oxygens of the phenol groups *This is the oxidation step* (It occurs only in the presence of an electron acceptor such as oxygen, which is reduced in the process) With the indicated electrons lost, only a single electron is shared by the hydrogens and oxygens of the phenol groups Since *two* shared electrons are required for stable bonds, the hydrogen is now very weakly

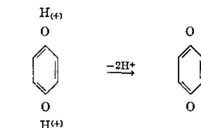
[*] The superscript ^{0} as in Cl^{0} indicates the uncharged atom It does not necessarily represent the uncharged molecule which in the case of chlorine is Cl_2 It is simpler for our purposes to deal here with single atoms

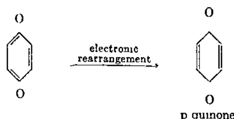
held and in step 2 spontaneously leaves the molecule as a hydrogen ion *This is the dehydrogenation step* The loss of hydrogen by a molecule is thus not itself oxidation but is rather the consequence of oxidation Step 3



L

1


2


3


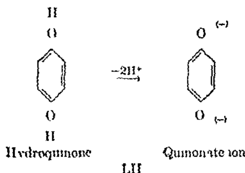
LI

represents only a rearrangement of electrons into a more stable configuration

It is important to distinguish between dehydrogenation and ionization

In the case of hydroquinone, dehydrogenation is as has been indicated the loss of two electrons followed by the loss of two hydrogen ions while ionization would be the loss of hydrogen ions without loss of electrons leaving a charged negative ion (formula LII) Ionization is thus not an oxidation reduction process though the extent to which it is present may influence the ease of oxidation In the case of hydroquinone, the preliminary *dissociation of hydrogen ion encourages oxidation since electrons are more* easily removed from the negatively charged quinonate ion than from the neutral hydroquinone itself It is for this reason that substances such as hydroquinone are particularly liable to oxidation at alkaline pH

In the oxidation of ferrous ion by chlorine, electrons move from the *ferrous ion to the chlorine but not vice versa Similarly, carbon can be* completely oxidized by oxygen to carbon dioxide, and hydroquinone to



Hydroquinone   Quinonate ion

LII

p quinone, the reactions being not measurably reversible This is so because of the considerably greater attraction for electrons on the part of chlorine and oxygen as compared with the other substances mentioned It is possible to measure by electrochemical methods the relative attraction various substances have for electrons (15) and this measure is termed the *oxidation potential*

The oxidation potential is always given for a *system* which includes the reduced and oxidized form of a single substance, ... ced form being conventionally placed first Since the absolute va... dation poten tials can not be measured, th... n potential o... en/hydrogen ion system is arbitrarily set ... d other pot... related to it. Systems in which the redu... a greater t... e electrons than ... lrogen have ... on potent... uch the r... orm hea... to lose ... ve oxidat... tials ... as one ... k of oxi... ntial ... asing positi... tems ... wa

retain electrons and remain in the reduced forms The oxidized forms of such systems with their great tendency to gain electrons and be reduced, are therefore *oxidizing substances* At the other end of the scale are located the *reducing substances* It is important to realize that both these terms are relative The hydrogen hydrogen ion system is an oxidizing substance with respect to potassium (explosively so) but is a reducing substance with respect to oxygen Oxygen in turn usually considered an oxidizing substance (it gave its very name to the process), is a reducing substance with respect to fluorine In general, if two redox systems are mixed, electrons will flow in the direction of the higher positive or smaller negative potential In other words a given redox system will oxidize and be reduced by any other system with a less positive or more negative potential

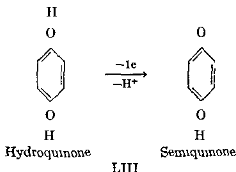
In the cases cited in the earlier part of this section for instance, the chloride ion/chlorine system has an oxidation potential of 1 3583 volts while that of the ferrous ion ferric ion system is 0 771 Again the oxidation potential of water/oxygen is 0 815 while that of hydroquinone/p-quinone is 0 6994 The oxidation potentials of many systems of biochemical interest have been determined and in Lardy (2) one can find a listing of 231 of these In some of these systems the oxidized and reduced forms are of different colors (as in 2 6-dichlorophenolindophenol), or one form is colored and one is not (as in methylene blue) Such systems are very useful as oxidation reduction indicators if their oxidation potential is intermediate between those of the two systems whose interaction is being studied The principle is quite analogous to the use of pH indicators In using oxidation potentials it is always important to remember that they vary with the temperature and pH of the system

Although many oxidation reduction reactions go virtually to completion pairs of systems which have oxidation potentials close to one another yield equilibria in which measurable quantities of both the oxidized and reduced forms of the systems concerned may exist Such systems can respond readily to the addition of either oxidizing or reducing systems and are referred to as being *poised* just as certain acid base systems are *buffered* The living cell is such a poised system and much of its behavior can be explained on the basis of that fact

Semiquinone theory of oxidation The oxidizing reactions with which physicians are primarily concerned are those of oxygen on foodstuffs If we consider the oxidizing properties of molecular oxygen in this connection we are brought face to face with an apparent contradiction Oxygen is the second most electronegative element known yielding first place only to fluorine (24) It is more electronegative than chlorine as is evidenced by the fact that oxygen in combination with carbon or hydrogen can attract the shared electrons sufficiently to accumulate enough charge to form

hydrogen bonds, whereas chlorine attached to carbon or hydrogen can not so successfully compete for electrons (This ability on the part of oxygen to form hydrogen bonds while chlorine can not is not entirely due to the greater electronegativity of the former atom Oxygen is the smaller atom and can approach closer to the hydrogen so that there is a stronger attraction between the two unlike charges) One would assume from this that oxygen would be the stronger oxidizing agent since that is what is meant by this greater tendency to gain electrons Yet in actual practice the reverse seems to be true Chlorine (in the presence of light) will react readily with hydrogen or hydrocarbons at room temperature, while oxygen requires elevated temperatures What is the answer to this apparent contradiction?

In the previous section we have considered oxidations involving oxygen and one involving chlorine, and one difference between them—electronically



Hydroquinone Semiquinone

LIII

speaking—stands out In the oxidation of ferrous ion by chlorine, one electron is transferred, since chlorine requires only one to make up its octet In the oxidation of hydroquinone by oxygen, *two* electrons are transferred since oxygen requires that many for octet formation There is evidence (21) that the two electron transfer involved in oxidations by oxygen is accomplished one electron at a time This means that in the case of hydroquinone there must be an intermediate stage which is neither hydroquinone nor *p* quinone, but something in between This intermediate is known as a *semiquinone* and shown in formula LIII Note that the semiquinone has lost both an electron and a hydrogen ion, and is therefore not charged It must not be confused with the quinonate ion existing in strongly alkaline solutions in which only a hydrogen ion has been lost and which has a unit negative charge in consequence

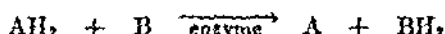
All hydrocarbon derivatives, when oxidized by oxygen, pass through such a one electron loss semiquinone stage The name is applied even to intermediates of compounds which do not form quinones on oxidation The effect was first studied in quinones and their reduced derivatives, in

certain of which it is even possible to demonstrate visually the presence of such intermediates The significance of this stage in oxidation by oxygen rests on the fact that the semiquinone is an extremely unstable structure the formation of which requires an input of energy In other words, semiquinone formation involves an increase of free energy and is therefore not itself a spontaneous reaction Once formed as through the application of heat energy, the semiquinone readily loses the second electron, the loss of free energy in this second step more than making up for the gain in the first so that the overall reaction is spontaneous For this reason a candle *once lit* will continue to burn freely Similarly, oxygen and hydrogen combine at room temperature but at infinitesimal rates since only the rare molecules which collide with sufficient energy to form the semiquinone stage can proceed to the final oxidation (It is evident from the foregoing discussion that nitrogen which requires a *three*-electron shift to form its octet will behave in practice almost like an inert gas, despite the fact that it is about as electronegative as chlorine and since it is smaller can form hydrogen bonds)

Oxidizing Enzymes

Oxidizing enzymes catalyze the transfer of electrons from one substance to another Since biological oxidations in general usually involve the C H and the O H bond, the transfer of electrons involves the transfer of hydrogen as well Although it is not the basic process, the hydrogen transfer is so conspicuous a part of the reaction that in Baldwin's classification cited earlier in the chapter oxidizing enzymes are classified with the transferring enzymes a pair of hydrogens being the chemical group transferred

If we consider two redox systems AH_2/A and BH_2/B, of which the latter has the higher oxidation potential then the general reaction catalyzed by an oxidizing enzyme would be



$$AH_2 + B \xrightarrow{\text{enzyme}} A + BH_2$$

AH_2 would represent the *metabolite* or the *hydrogen donor* while B is the *hydrogen acceptor* It is possible to classify the various oxidizing enzymes according to the nature and specificity of the hydrogen acceptors involved Three categories can be listed

1 Enzymes which can utilize organic compounds as hydrogen acceptors These are the *dehydrogenases*

2 Enzymes which specifically require molecular oxygen as the hydrogen acceptor These are the *oxidases*

3 Enzymes which specifically require hydrogen peroxide as the hydrogen acceptor These are the *peroxidases*

These classes can be further differentiated by the fact that the oxidases

and peroxidases possess metal containing prosthetic groups, whereas the dehydrogenases do not

Dehydrogenases In the presence of certain tissue extracts a variety of organic acids, aldehydes, and alcohols can be oxidized if an appropriate hydrogen acceptor is also included in the system Pure solutions of succinic acid are stable in the presence of either methylene blue or homogenized liver tissue In the presence of both methylene blue and tissue, however, succinic acid is oxidized to fumaric acid This can be demonstrated by the fact that in the process methylene blue is reduced to a colorless compound If methylene blue is mixed with succinic acid, none of the blue color is lost even over extended periods If tissue extract is added as well, decolorization takes place rapidly Again, if methylene blue and tissue extract are mixed no decolorization takes place until succinic acid is added from the side bulb In such experiments it is usual to maintain a nitrogen atmosphere since in the presence of oxygen reduced methylene blue is spontaneously re-oxidized to methylene blue and the bleaching effect is thus minimized or entirely lost The succinic acid dehydrogenase which catalyzes this reaction will not itself function except in the presence of DPN as coenzyme Apparently the reaction can thus be broken up into two steps each of which involves the transfer of two hydrogen atoms

1 Succinic acid is oxidized to fumaric by DPN which is reduced to DPN H_2,

2 DPN H_2 is re oxidized to DPN by methylene blue which is itself reduced to the colorless leucomethylene blue

Substances such as DPN which in a process of this sort first gain hydrogen and then lose it are termed *hydrogen carriers* Such hydrogen carriers shuttle repeatedly between their oxidized and reduced forms so that one molecule can handle indefinite numbers of metabolite molecules Such a substance need be present only in small quantities in order to perform its function Among the coenzymes, DPN, TPN, and the alloxazine nucleotides act in this fashion Other widely distributed hydrogen carriers in living tissue whose functions are not yet fully defined include ascorbic acid, the tocopherols, and glutathione

Of the two steps listed in the enzymatic oxidation of succinic acid, there is a lack of specificity in the second which is characteristic of the dehydrogenases With few exceptions any redox system with a suitable oxidation potential can substitute for methylene blue as a hydrogen acceptor Oxygen itself is the most important exception It can not accept the hydrogen from DPN directly, presumably because of the sluggishness involved in the semiquinone step which was discussed in the previous section Some dehydrogenases show even less specificity with respect to hydrogen acceptance In the case of xanthine dehydrogenase, in which FAD is the coenzyme

oxidation of xanthine to uric acid can take place not only in the presence of such oxidizing agents as indophenol, alloxan chlorates, iodine, nitrates nitrobenzene, permanganate, quinone, methylene blue, and hydrogen per oxide—but in the presence of molecular oxygen as well Dehydrogenases can be divided into aerobic and anaerobic according to whether they can or can not use molecular oxygen as a possible hydrogen acceptor

Aerobic dehydrogenases can be distinguished from true oxidases not only by the fact that the former can utilize substances other than molecular oxygen as the hydrogen acceptor, while the latter is restricted to oxygen, but also by the fact that the use of oxygen by the former results in its reduction to hydrogen peroxide while by the latter it is reduced to water The difficulty of attempting to make any enzyme classification can be exemplified by uricase, also known as uric acid dehydrogenase and urico oxidase This enzyme catalyzes the oxidation of uric acid only in the pres ence of molecular oxygen as hydrogen acceptor It can not however, be considered a true oxidase, since the oxygen is reduced to hydrogen peroxide Uricase does not occur in human tissue and is of medical interest only in a negative sense since its absence from human tissue makes gout possible (see Chapter 7)

Oxidases. The characteristic structural distinction between oxidases and dehydrogenases is the presence of a metal prosthetic group in the former Two types of oxidases may be recognized, depending upon the nature of the metal involved The copper oxidases include such enzymes as tyrosinase, catecholase, and ascorbic acid oxidase These are primarily plant enzymes

The iron oxidase, *cytochrome oxidase*, occurs in nearly all living cells, including the human It contains a heme prosthetic group and displays a high degree of specificity, since it catalyzes the oxidation of the cytochromes only (26) The cytochromes are themselves proteins, containing heme prosthetic groups, and capable of existing in oxidized and reduced states, so that they may function as hydrogen carriers Spectroscopically, the cytochromes have been differentiated into several closely related com pounds, usually designated as cytochrome a_1, a_2 b, and c Of these only *cytochrome c* is easily extractable, the others along with cytochrome oxidase itself being tightly bound to the cell structure itself Of the cyto chromes, cytochrome c is the one which has been most studied in con nection with cytochrome oxidase Its relationship to the enzyme is pre cisely that of DPN to succinic acid dehydrogenase Functionally, it is the coenzyme of cytochrome oxidase though since it is a protein it differs chemically from other coenzymes in that it is not thermostable and can not be dialyzed away The role of the other cytochromes is still doubtful *Cytochrome b* is autoxidizable and does not require the presence of cyto chrome oxidase It is responsible for a small amount of respiration (about

10 per cent) which proceeds even under cyanide inhibition This may represent a possible alternate respiratory route which, unfortunately, has not developed to the point where it alone can satisfy the needs of the cell

Peroxidases. The peroxidases are a smaller group of enzymes than either of those discussed above Peroxidases are usually quite unspecific with regard to metabolites catalyzing the oxidation of many phenols and aromatic amines Peroxidases are heme containing enzymes which occur primarily in plants The only occurrence in animal tissues reported are in milk and in leucocytes The two are not identical

Catalase. Catalase is widely distributed in animal tissues and many microorganisms The reaction it catalyzes may be looked upon as the transfer of hydrogen from one molecule of hydrogen peroxide to another to yield oxygen and water From this viewpoint it is a peroxidase (a peroxide peroxidase, in fact) which group it further resembles by being a heme containing protein

MULTIPLE ENZYME SYSTEMS

Up to this point we have discussed enzymes as single entities, and in so doing we have indulged in an oversimplification It can not be too often stressed that the living cell is a unit and that within it no one enzyme acts independently of the others

The Cellular Respiratory Chain

As an example we may take the oxidizing enzymes discussed in the previous section We have mentioned the dehydrogenases which require the presence of a hydrogen acceptor which may not be oxygen and which indeed, sometimes can not be On the other hand, there are the oxidases which require oxygen and oxygen only as the hydrogen acceptor Two questions arise Why does the cell require both kinds of oxidizing enzymes? And, since substances such as methylene blue and ferricyanide ion do not occur in the cells, what is the natural hydrogen acceptor for the dehydrogenases?

The second question may be answered at once if we consider that the two enzyme systems are complementary The natural hydrogen acceptor of a dehydrogenase is the cytochrome oxidase system, either directly or indirectly through other dehydrogenases For example, a known three-component system in cellular respiration would be

1 An enzyme such as succinic acid dehydrogenase, lactic acid dehydrogenase or any of those for which DPN or TPN acts as coenzyme

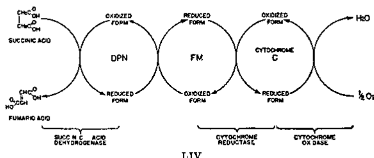
2 Cytochrome reductase (14) for which FM is the coenzyme

3 Cytochrome oxidase for which cytochrome c is the coenzyme

What actually occurs in this system is that a "bucket brigade" for hy

drogen atoms (or more fundamentally, for electrons) is set up The metabolite succinic acid, lactic acid, or any of numerous other products of food metabolism, is oxidized in the presence of the specific dehydrogenase, passing two atoms of hydrogen to DPN, which is reduced in the process The reduced DPN passes the hydrogen to the FM coenzyme of cytochrome reductase and is thus re-oxidized while the latter is reduced The reduced FM passes the hydrogens to cytochrome c and is thus re oxidized while the latter is reduced Finally, the reduced cytochrome c in the presence of cytochrome oxidase passes its hydrogen to molecular oxygen and is re oxidized while the latter is reduced to water

Baldwin (3) has devised an ingenious method of representing such enzymatic bucket brigades which is a great help in visualizing the process described above (formula LIV)

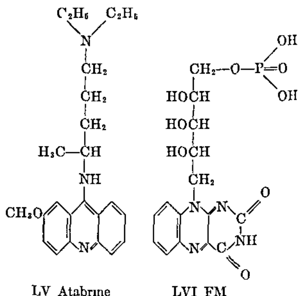


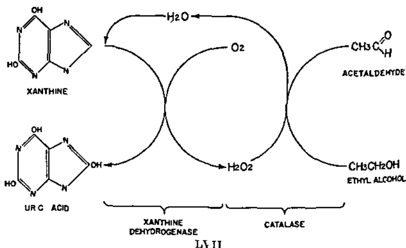
LIV

Observe that the three coenzymes travel in circles and are not used up in the process The only substances that are used up are the succinic acid (or other such metabolite) at one end of the chain and oxygen at the other From this fact it can be seen that extremely small quantities of the coenzymes are sufficient for the purpose and that similarly small quantities of coenzyme poisons will end the respiratory process and with it life itself Such substances as HCN through the formation of stable iron complexes interfere with the oxidation of reduced cytochrome c, and stop that particular "wheel" and the cell, or organism, dies The wheel can be just as effectively and fatally stopped through interferences with the reduction of oxidized cytochrome c as is accomplished by many of the narcotics such as the barbiturates Nor are the other components of the system less vulnerable The action of atabrine (formula LV) on the malaria parasite is thought to be the result of its competitive inhibition of FM (formula LVI), which it resembles somewhat (14)

The position of cytochrome oxidase at the oxygen end of the chain should be noted It has already been stated that its function is to reduce

oxygen without formation of hydrogen peroxide, which is poisonous to biological systems It has been suggested that in the case of the aerobic dehydrogenases which do produce hydrogen peroxide by the reduction of





LVII

oxygen, a coupling with catalase takes place so that not only is the harmful substance destroyed but use is made of the energy released in the decomposition to oxidize such substances as the simple alcohols (16) Such a catalase bound system could be represented as in formula LVII

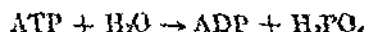
We have not yet answered the question as to why various kinds of oxidizing enzymes are required by the cell or in other words why oxidations must be conducted in this stepwise fashion. The answer to this is that in each individual step the difference in oxidation potential is comparatively small so that the whole represents a well poised system the action of which can be more delicately controlled by the concentration of nutrient metabolites and the partial pressure of oxygen in the cells. The difference between bucket brigade oxidation and one step oxidation may therefore be compared with that between sliding downhill and falling off a precipice.

Phosphate-Linked Chains

Phosphate transfer to and from ADP and ATP (see Chapter 3) is not only a means of transferring and storing chemical energy but also by the cyclic nature of the process links different but related enzyme reactions. Phosphate linked enzyme systems are of particular importance in carbohydrate metabolism (Chapter 12). Some inspection of the principles involved will be useful at this point however.

Glucose in the process of absorption into the intestinal mucosal cells is converted to glucose 6 phosphate at the expense of ATP which loses a high-energy phosphate bond and becomes ADP. This reaction is catalyzed by the enzyme glucokinase. Under physiological conditions this reaction is irreversible[4] since to reverse it directly would require an input of energy equivalent to that released by the original conversion of a high energy phosphate bond to one of low energy. In order to regain free glucose from the glucose 6 phosphate the mucosal cell hydrolyzes it in the presence of a phosphatase to form glucose and phosphoric acid. These two reactions can be represented by the Baldwin system as shown in formula IV III.

Such a representation shows that in the two reactions described glucose and glucose 6 phosphate are not consumed. The over all reaction is

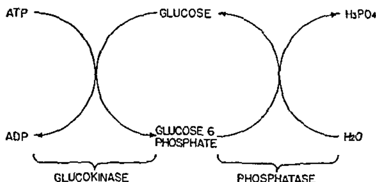


$$ATP + H_2O \rightarrow ADP + H_3PO_4$$

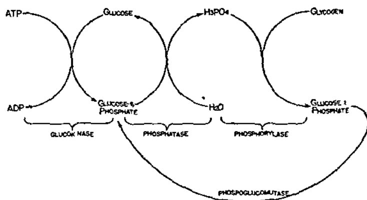
yet phosphoric acid does not accumulate, nor does ATP entirely disappear. They take part in other reactions both in the mucosal cells and elsewhere in the body. Let us take up phosphoric acid first. In the presence of phosphorylase it will convert glycogen to glucose 1 phosphate, which under

[4] Thermodynamically no reaction occurring in a closed system is truly irreversible under any conditions. It often happens however that the equilibrium point of a given reaction under given conditions is so immeasurably close to the point of absolute reaction completion that in any practical sense a simpler picture of prevailing conditions is given by considering such a reaction to be irreversible. Such a practice will be followed in this book without however implying any denial of the basic concepts of thermodynamics.

the influence of phosphoglucomutase can be converted by phosphate transfer within the molecule to glucose 6 phosphate Adding these reactions to the system, the Baldwin representation would be as shown in formula LIX It is not necessary at this point to concern ourselves with the renewal of glycogen and water, since both are obtained directly or indirectly from foodstuffs in ample quantity What is important, however, is the manner



LVIII

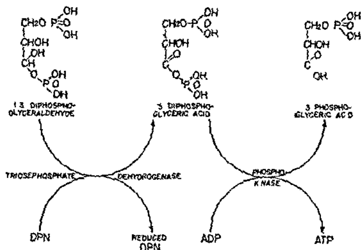


LIX

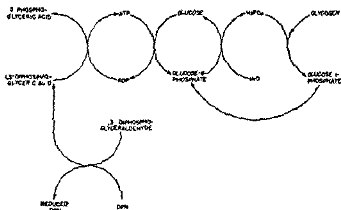
in which the high-energy phosphate bond is recreated and ADP reconverted to ATP

There are several physiological reactions involving oxidation, by which a low-energy phosphate bond is converted to one of high energy One such is the oxidation of 1-3-diphosphoglyceraldehyde to 1-3-diphosphoglyceric acid, under the influence of triosephosphate dehydrogenase, an enzyme utilizing DPN as coenzyme In the process a low energy ester phosphate is converted to a high energy acyl phosphate, the free energy gain being more than made up for by the free energy loss involved in the oxidation

1-3 Diphosphoglyceric acid can now transfer its high energy phosphate to ADP under the influence of a phosphokinase, forming ATP and itself being hydrolyzed to 3 phosphoglyceric acid These last two reactions can



LX

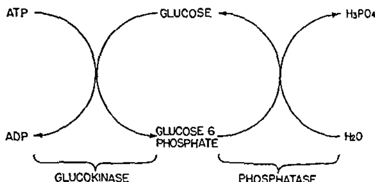


LXI

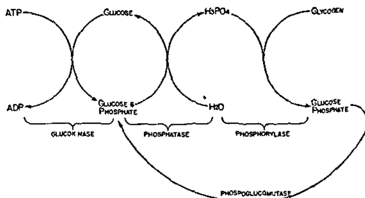
be represented as shown in formula LX This system, involving an oxidation can now be combined with the previous system involving phosphorylation through the ATP/ADP shuttle as shown in formula LXI

By proceeding in this manner still further it is possible to link up enzyme systems in ever more complicated fashion so that it is not too difficult

the influence of phosphoglucomutase can be converted by phosphate transfer within the molecule to glucose 6 phosphate Adding these reactions to the system, the Baldwin representation would be as shown in formula LIX It is not necessary at this point to concern ourselves with the renewal of glycogen and water since both are obtained directly or indirectly from foodstuffs in ample quantity What is important however, is the manner



LVIII

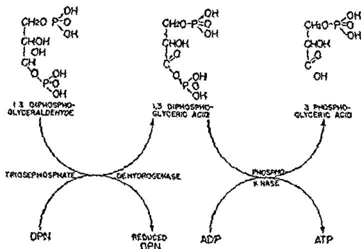


LIX

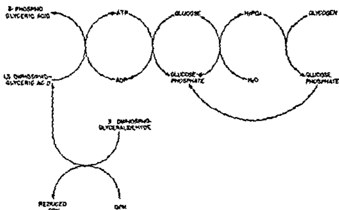
in which the high energy phosphate bond is recreated and ADP re verted to ATP

There are several physiological reactions involving oxidation, by w a low energy phosphate bond is converted to one of high energy One is the oxidation of 1 3-diphosphoglyceraldehyde to 1 3 diphosphogly acid, under the influence of triosephosphate dehydrogenase an enz utilizing DPN as coenzyme In the process a low energy ester phosp is converted to a high energy acyl phosphate, the free energy gain b more than made up for by the free energy loss involved in the oxidat

1-3 Diphosphoglyceric acid can now transfer its high energy phosphate to ADP under the influence of a phosphokinase, forming ATP and itself being hydrolyzed to 3 phosphoglyceric acid These last two reactions can



LX

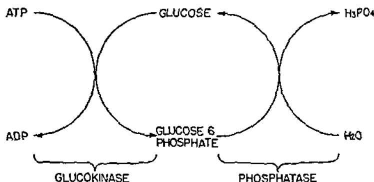


LXI

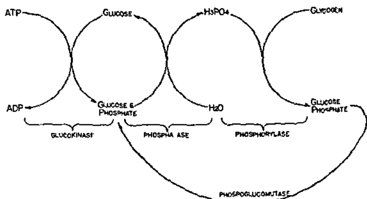
be represented as shown in formula LX This system, involving an oxidation, can now be combined with the previous system involving phosphorylation through the ATP/ADP shuttle as shown in formula LXI

By proceeding in this manner still further it is possible to link up enzyme systems in ever more complicated fashion so that it is not too difficult

the influence of phosphoglucomutase can be converted by phosphate *transfer* within the molecule to glucose 6 phosphate Adding these reactions to the system the Baldwin representation would be as shown in formula LIX It is not necessary at this point to concern our elves with the renewal of glycogen and water since both are obtained directly or *indirectly from foodstuffs* in ample quantity What is important however is the manner



LVIII



LIX

in which the high energy phosphate bond is recreated and ADP reconverted to ATP

There are several physiological reactions involving oxidation by which a low-energy phosphate bond is converted to one of high energy One such is the oxidation of 1-3 diphosphoglyceraldehyde to 1 3 diphosphoglyceric acid under the influence of triosephosphate dehydrogenase an enzyme utilizing DPN as coenzyme In the process a low energy ester phosphate is converted to a high energy acyl phosphate the free energy gain being more than made up for by the free energy loss involved in the oxidation

1-3-Diphosphoglyceric acid can now transfer its high energy phosphate to ADP under the influence of a phosphokinase, forming ATP and itself being hydrolyzed to 3 phosphoglyceric acid These last two reactions can

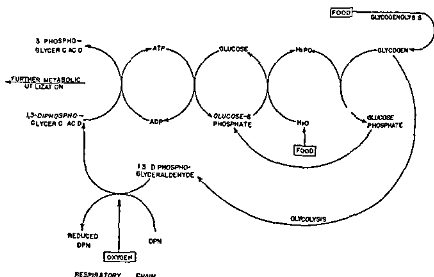
LX

LXI

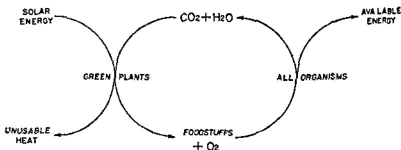
be represented as shown in formula LX. This system, involving an oxidation, can now be combined with the previous system involving phosphorylation through the ATP/ADP shuttle as shown in formula LXI

By proceeding in this manner still further it is possible to link up enzyme systems in ever more complicated fashion so that it is not too difficult

to imagine the entire cell as consisting of a single multi-enzyme system intricately interrelated and, in health at least, working with smooth precision Naturally, it is not a closed system Not all the components are renewed Some materials are used up and not reformed, while others are



LXII



LXIII

formed and not re used In the system just described glycogen is the source of 1 3-diphosphoglyceraldehyde and is itself formed from foodstuffs by processes to be described in Chapter 12 The system, moreover, is linked with the respiratory chain and, therefore, with the ultimate use of oxygen as an energy source through the DPN/DPN H_2 step (formula LXII) The over all change is thus

carbon and hydrogen of foodstuffs + oxygen →

carbon dioxide + water + usable energy

If green plants did not exist this over all reaction would be irreversible Fortunately, the photosynthetic process can reverse the reaction through the utilization of solar energy (formula LXIII)

The over all reaction now becomes

solar energy → available energy + unusable heat,

a relationship which is as far as we know unqualifiedly irreversible However the total supply of solar energy is as yet sufficient to last the human race for about ten to twenty billions of years

LOCATION OF ENZYMES IN CELLS

Understanding of enzymes and enzyme systems in the cellular economy can be enhanced if these are studied not merely as substances indiscriminately present in tissue or its extracts but as chemical entities organized in a definite manner within the cell Many techniques have been developed to locate concentrations of specific enzymes within the cell

Cytochemistry

An example of a cytochemical technique is the elegant method applied to the determination of the intracellular location of phosphatase (12) Tissue slices only a few thousandths of a millimeter in thickness are used In earlier work these slices were dehydrated by treatment with acetone and then embedded in warm paraffin Such treatment however will seriously denature many enzymes present More recently slices of tissue after removal from the organism are quickly frozen and then sectioned at low temperature without further treatment Sections so obtained are incubated with a substrate such as glyceryl phosphate in the presence of small quantities of an ion such as Ca^{++} capable of forming an insoluble phosphate The glyceryl phosphate is hydrolyzed by the phosphatase present in the tissue section and just at those points in the cell where the enzyme is located the concentration of free phosphate rises sharply Before diffusion of the phosphate ions can take place, they are precipitated by the calcium present as calcium phosphate The sections are then removed washed with distilled water and placed in solutions of cobalt acetate or lead acetate The calcium phosphate is converted to cobalt or lead phosphate Finally the sections are placed in a solution of ammonium sulfide so that the white phosphate is converted into the black cobalt sulfide or lead sulfide Under the microscope the original location of phosphatase within the cell can now be seen as dark brown or black patches

An example of the utilization of such a test in studying the content and distribution of acid phosphatase in cancerous cells and homologous normal tissue will be found in a paper by Lemon and Wisseman (19) Interested

students are referred to a monograph by Glick (11), covering all phases of cytochemistry

Separation of Cell Fragments

Another increasingly prominent technique for determining the intracellular location of enzymes may be summarized as the subdivision of cells into fractions containing specific organelles Thus, Dounce (8) has devised methods whereby cell nuclei can be isolated free of any significant cytoplasmic admixture This is done, generally speaking, by homogenizing tissue at low temperature in the presence of citric acid solution By this procedure, the cell membrane is disrupted while the nuclear membrane remains intact The nuclei can then be separated from the cytoplasmic debris by filtration Such nuclei can be tested for enzyme activity In this manner, it has been found that many nuclei are deficient in oxidative systems, succinic acid dehydrogenase, for instance, being lacking, and are rich in such enzymes as arginase, phosphatase, and catalase Differences in the nuclei of various tissues have been detected Thus liver cell nuclei are very rich in arginase, which is absent in kidney cell nuclei

A still finer separation of cellular components can be brought about by *differential centrifugation* (6) In this method, cellular homogenates or extracts are centrifuged in several stages in which the centrifugal force is increased each time At each stage, the sediment is collected In this way heavy particles are first collected and then successively lighter particles until only the cellular sap remains behind By differential centrifugation chromosomes can be isolated from nuclei and mitochondria, microsomes and miscellaneous granules of glycogen, ferritin, or melanin from the cytoplasm These various subcellular fractions are referred to generally as *particulates*

Of the particulates, the mitochondria have been most thoroughly studied They are composed largely of nucleoprotein and phospholipid Most or all of the cytochrome oxidase and succinic acid dehydrogenase of the cell is located in the mitochondria In general, mitochondria are rich in enzymes involved in aerobic oxidation and in oxidative phosphorylation, together with associated coenzymes such as FAD (18) Enzymes controlling glycolytic reactions are not located here It is tempting to consider such granules to be "macromolecules" in which various enzymes are organized about a nucleic acid framework to form the systems discussed earlier in the chapter The *cyclophorase* system (13) which catalyzes various stages in the metabolism of carbohydrates (see Chapter 12) is thought to exist as such a macromolecule An over all discussion of the chemistry and enzymology of cellular particulates can be found in Sumner and Myrback (9)

THEORIES OF ENZYME ACTION

The function of an enzyme, or indeed of any catalyst, energetically speaking, is to reduce the *energy of activation* of a reaction. Even where a reaction, under given conditions, is thermodynamically possible and hence spontaneous, there is always an intermediate stage during which the reaction has begun but is not yet complete where the free energy content is higher than at either end of the reaction. This energy 'hump' varies in height. In cases of oxidation of organic compounds by oxygen, the intermediate stage is represented by semiquinone formation and the energy of activation is quite high. In other cases the energy of activation may be so low that the ordinary thermal agitation of molecules at room temperature supplies sufficient kinetic energy for the reaction to proceed readily.

Exactly how an enzyme manages to reduce the energy of activation of a reaction is not yet clear. To say that the enzyme itself supplies the extra energy required from some mysterious store within itself begs the question, since it would only raise the necessity for explaining the source of the enzyme's nearly inexhaustible energy supply. Rather, by analogy with what has been learned concerning inorganic catalysts, it is tempting to consider enzymatic action as a surface phenomenon.

This may be made clearer if we consider that the ordinary uncatalyzed reaction results from the random collisions of molecules under the influence of thermal energy. For two individual molecules to react they must not only strike one another with sufficient energy for activation, but in some reactions they must come together with the proper orientation as well. In the case of the oxidation of hydroquinone by oxygen, it is reasonable to suppose that for the reaction to proceed the oxygen molecule must strike one of the two hydroxy groups. Collision with other parts of the benzene ring would result in an ineffectual rebound. In the case of reactions between even more complicated molecules, as is the rule in most of the systems that concern the physician, the importance of orientation is increased. (The student is warned that this discussion of the role played by orientation of collisions in reactions is metaphoric and not intended to be a rigorous picture of what is undoubtedly a much more complex phenomenon.)

The enzyme surface may be specifically designed to decrease the importance of chance in molecular orientation. To be sure, the substrate strikes the enzyme in the first place as a result of random motion, but once having collided with the enzyme it may form an enzyme-substrate complex, in which the substrate, as a result of the characteristics of the enzyme surface, is restricted to a certain orientation. If this orientation is such that a specific bond is peculiarly exposed to the bombardment of water molecules, while others are more or less protected, a hydrolysis would take place not

students are referred to a monograph by Glick (11), covering all phases of cytochemistry

Separation of Cell Fragments

Another increasingly prominent technique for determining the intracellular location of enzymes may be summarized as the subdivision of cells into fractions containing specific organelles Thus Dounce (8) has devised methods whereby cell nuclei can be isolated free of any significant cytoplasmic admixture This is done generally speaking by homogenizing tissue at low temperature in the presence of citric acid solution By this procedure the cell membrane is disrupted while the nuclear membrane remains intact The nuclei can then be separated from the cytoplasmic debris by filtration Such nuclei can be tested for enzyme activity In this manner it has been found that many nuclei are deficient in oxidative systems succinic acid dehydrogenase for instance being lacking and are rich in such enzymes as arginase phosphatase, and catalase Differences in the nuclei of various tissues have been detected Thus liver cell nuclei are very rich in arginase which is absent in kidney cell nuclei

A still finer separation of cellular components can be brought about by *differential centrifugation* (6) In this method, cellular homogenates or extracts are centrifuged in several stages in which the centrifugal force is increased each time At each stage the sediment is collected In this way heavy particles are first collected and then successively lighter particles until only the cellular sap remains behind By differential centrifugation chromosomes can be isolated from nuclei and mitochondria microsomes and miscellaneous granules of glycogen ferritin or melanin from the cytoplasm These various subcellular fractions are referred to generally as *particulates*

Of the particulates the mitochondria have been most thoroughly studied They are composed largely of nucleoprotein and phospholipid Most or all of the cytochrome oxidase and succinic acid dehydrogenase of the cell is located in the mitochondria In general mitochondria are rich in enzymes involved in aerobic oxidation a [illegible] together with associated coenzymes such [illegible] lytic reactions are not located he[illegible] to be macromolecules in which various enzymes are organized about a nucleic acid framework to form the systems discussed earlier in the chapter The *cyclophorase* system (13) which catalyzes various stages in the metabolism of carbohydrates (see Chapter 12) is thought to exist as such a macromolecule An over all discussion of the chemistry and enzymology of cellular particulates can be found in Sumner and Myrback (9)

THEORIES OF ENZYME ACTION

The function of an enzyme (as indeed of any catalyst, energetically speaking) is to reduce the *energy of activation* of a reaction. Even where a reaction, under given conditions, is thermodynamically possible and hence spontaneous, there is always an intermediate stage during which the reaction has begun but is not yet complete where the free energy content is higher than at either end of the reaction. This energy "hump" varies in height. In cases of oxidation of organic compounds by oxygen, the intermediate stage is represented by semiquinone formation and the energy of activation is quite high. In other cases the energy of activation may be so low that the ordinary thermal agitation of molecules at room temperature supplies sufficient kinetic energy for the reaction to proceed readily.

Exactly how an enzyme manages to reduce the energy of activation of a reaction is not yet clear. To say that the enzyme itself supplies the extra energy required from some mysterious store within itself begs the question, since it would only raise the necessity for explaining the source of the enzyme's nearly inexhaustible energy supply. Rather, by analogy with what has been learned concerning inorganic catalysts, it is tempting to consider enzymatic action as a surface phenomenon.

This may be made clearer if we consider that the ordinary uncatalyzed reaction results from the random collisions of molecules under the influence of thermal energy. For two individual molecules to react they must not only strike one another with sufficient energy for activation, but in some reactions they must come together with the proper orientation as well. In the case of the oxidation of hydroquinone by oxygen, it is reasonable to suppose that for the reaction to proceed the oxygen molecule must strike one of the two hydroxy groups. Collision with other parts of the benzene ring would result in an ineffectual rebound. In the case of reactions between even more complicated molecules, as is the rule in most of the systems that concern the physician, the importance of orientation is increased. (The student is warned that this discussion of the role played by orientation of collisions in reactions is metaphoric and not intended to be a rigorous picture of what is undoubtedly a much more complex phenomenon.)

The enzyme surface may be specifically designed to decrease the importance of chance in molecular orientation. To be sure, the substrate strikes the enzyme in the first place as a result of random motion, but once having collided with the enzyme it may form an enzyme-substrate complex, in which the substrate, as a result of the characteristics of the enzyme surface, is restricted to a certain orientation. If this orientation is such that a specific bond is peculiarly exposed to the bombardment of water molecules, while others are more or less protected, a hydrolysis would take place not

students are referred to a monograph by Glick (11), covering all phases of cytochemistry.

Separation of Cell Fragments

Another increasingly prominent technique for determining the intracellular location of enzymes may be summarized as the subdivision of cells into fractions containing specific organelles. Thus Dounce (8) has devised methods whereby cell nuclei can be isolated free of any significant cytoplasmic admixture. This is done, generally speaking, by homogenizing tissue at low temperature in the presence of citric acid solution. By this procedure the cell membrane is disrupted while the nuclear membrane remains intact. The nuclei can then be separated from the cytoplasmic debris by filtration. Such nuclei can be tested for enzyme activity. In this manner it has been found that many nuclei are deficient in oxidative systems (succinic acid dehydrogenase, for instance, being lacking) and are rich in such enzymes as arginase, phosphatase, and catalase. Differences in the nuclei of various tissues have been detected. Thus liver cell nuclei are very rich in arginase, which is absent in kidney cell nuclei.

A still finer separation of cellular components can be brought about by *differential centrifugation* (6). In this method, cellular homogenates or extracts are centrifuged in several stages in which the centrifugal force is increased each time. At each stage the sediment is collected. In this way heavy particles are first collected and then successively lighter particles until only the cellular sap remains behind. By differential centrifugation, chromosomes can be isolated from nuclei and mitochondria, microsomes, and miscellaneous granules of glycogen, ferritin, or melanin from the cytoplasm. These various subcellular fractions are referred to generally as *particulates*.

Of the particulates, the mitochondria have been most thoroughly studied. They are composed largely of nucleoprotein and phospholipid. Most or all of the cytochrome oxidase and succinic acid dehydrogenase of the cell is located in the mitochondria. In general, mitochondria are rich in enzymes involved in aerobic oxidation and in oxidative phosphorylation, together with associated coenzymes such as FAD (18). Enzymes controlling glycolytic reactions are not located here. It is tempting to consider such granules to be macromolecules in which various enzymes are organized about a nucleic acid framework to form the systems discussed earlier in the chapter. The *cyclophorase* system (13) which catalyzes various stages in the metabolism of carbohydrates (see Chapter 12) is thought to exist as such a macromolecule. An over-all discussion of the chemistry and enzymology of particulates can be found in Sumner and Myrbäck (9).

THEORIES OF ENZYME ACTION

The function of an enzyme, as indeed of any catalyst, energetically speaking, is to reduce the *energy of activation* of a reaction. Even where a reaction, under given conditions, is thermodynamically possible and hence spontaneous, there is always an intermediate stage during which the reaction has begun but is not yet complete where the free energy content is higher than at either end of the reaction. This "energy bump" varies in height. In cases of oxidation of organic compounds by oxygen, the intermediate stage is represented by semiquinone formation, and the energy of activation is quite high. In other cases the energy of activation may be so low that the ordinary thermal agitation of molecules at room temperature supplies sufficient kinetic energy for the reaction to proceed readily.

Exactly how an enzyme manages to reduce the energy of activation of a reaction is not yet clear. To say that the enzyme itself supplies the extra energy required from some mysterious store within itself begs the question, since it would only raise the necessity for explaining the source of the enzyme's nearly inexhaustible energy supply. Rather, by analogy with what has been learned concerning inorganic catalysts, it is tempting to consider enzymatic action as a surface phenomenon.

This may be made clearer if we consider that the ordinary uncatalyzed reaction results from the random collisions of molecules under the influence of thermal energy. For two individual molecules to react they must not only strike one another with sufficient energy for activation, but in some reactions they must come together with the proper orientation as well. In the case of the oxidation of hydroquinone by oxygen, it is reasonable to suppose that for the reaction to proceed the oxygen molecule must strike one of the two hydroxyl groups. Collision with other parts of the benzene ring would result in an ineffectual rebound. In the case of reactions between even more complicated molecules, as is the rule in most of the systems that concern the physician, the importance of orientation is increased. (The student is warned that this discussion of the role played by orientation of collisions in reactions is metaphoric and not intended to be a rigorous picture of what is undoubtedly a much more complex phenomenon.)

The enzyme surface may be specifically designed to decrease the importance of chance in molecular orientation. To be sure, the substrate strikes the enzyme in the first place as a result of random motion, but once having collided with the enzyme it may form an enzyme-substrate complex, in which the substrate, as a result of the characteristics of the enzyme surface, is restricted to a certain orientation. If this orientation is such that a specific bond is peculiarly exposed to the bombardment of water molecules, while others are more or less protected, a hydrolysis would take place not

only more rapidly than in the absence of enzymatic interference, but also more specifically It is even conceivable that an enzyme surface would not only have a tendency to orient its substrate, but also the other reagent, such as a water molecule, a phosphoric acid molecule, an appropriate coenzyme, or a molecule of ATP

As a result of the characteristic specificity of enzyme action, much has been made of the so called "lock and key' hypothesis Here, the enzyme surface is thought to be constructed in such a manner as to allow a substrate or a coenzyme to fit snugly as a key would fit into a lock The phenomenon of competitive inhibition would be easily explained in this manner A compound similar to the one for which the enzyme surface is adapted might be able to squeeze in, yet not snugly enough to allow proper orientation just as a slightly wrong key might be forced into a lock and remain motionless (or, worse, break off) when an attempt is made to turn it

The simplest view of such a lock and key mechanism would consider the atoms themselves as being so arranged as to form a depression in the surface into which the substrate molecule or a portion thereof will just fit Another view would consider the arrangement of surface charge on the enzyme to be such that the electrostatic attraction between it and the substrate molecule in a certain orientation is greater than it would be for any other orientation or any other molecule

Geissman (10) considers the protein nature of the enzyme to be the important consideration in any theory of enzyme action The repetitive peptide chain and the periodic hydrogen bridge form channels along which electrons can pass freely The participation of such electron channels in the reaction being catalyzed tends to distribute the energy of the intermediate over the entire protein In this fashion the intermediate (semiquinone or otherwise) is rendered more stable and the energy of activation is lowered

REFE[illegible]

1 ALDRIDGE W N Beryllium and alk [illegible] atase Natu[illegible] 950
2 ANDERSON, I , [illegible] ER, G W E [illegible] dation redu[illegible] ls, in LARDY H [illegible] ratory [illegible] edit pp [illegible] polis Minn Burg[illegible] Co , I[illegible]
3 BALDWIN E J [illegible] s of B[illegible] mbridge, [illegible] r Ige University
4 BERGMANN, M [illegible] on [illegible] zymes [illegible] 1, 2 49–68 194[illegible]
5 BORDNER C [illegible] eres [illegible] nase J Am[illegible]
6 CLAUDE A Th[illegible] bution of nuc[illegible] 19–29, 1941

7 DAWSON C R AND MALLETTE M F The copper proteins Advances Prot Chem 2 179-248 1945
8 DOUNCE A L Enzyme stud es of isolated cell nuclei of rat liver J B ol Chem 147 685-698 1943
9 DOUNCE A L Cytochem cal f undations of enzyme chemistry in SUMNER J B AND MYRBACK K edits *The Enzymes* Vol I Part I p p 187 266 New York Academic Press 1950
10 GEISSMAN T A Theory of the mechan sm o enzyme action Quart Rev Biol 24 309-397 1949
11 GLICK D *Techniques of H sto nd Cytochem st y* New York Interscience Pub l shers 1949
12 GOMORI G D stribution of ac d phosphatase in the tissues under normal and under pathologic conditions Arch Path 32 189-199 1941
13 GREEN D E The cyclophorase system in I D ALL J T ed *Enzymes and Enzyme Systems* pp 15-46 Cambridge Mass Harvard Univers ty Press 1951
14 HAAS E The effect of atal rine and quinine on isolated resp ratory enzymes J B ol Chem 155 321-331 1944
15 JOHNSON M J Oxidation reduction potentials n LARDY H A ed *Respiratory Enzymes* rev edit pp 58-70 Minneapol s Minn Burgess Publ shing Co 1949
16 KEILIN D AND HARTREE E F Propert es of catalase Cata s s of coupled oxidation of alcohols B ochem J 39 293 301 1945
17 KORNBERG A AND PRICER W E On the structure of triphosp hopyridine nucleotide J Biol Chem 186 557-567 1950
18 LEHNINGER A L The organized respiratory activity of isolated rat liver mitochondria in EDSALL J T ed *Enzymes and Enzyme Systems* pp 1 14 Cambridge Mass Harvard University Press 1951
19 LEMON H M AND WISSEMAN C L JR Acid phosphomonoesterase activity of human neoplastic tissue Science 109 233 235 1949
20 LIPMANN F *et al* Isolation of coenzyme A J Biol Chem 186 235-243 1950
21 MICHAELIS L in GREEN D E ed *Currents n B ochem cal Research* pp 207 228 New York Interscience Publishers 1946
22 MILLER W H *et al* A new method for the measurement of tyrosinase activity II Catecholase activity based on the initial reaction velocity J Am Chem Soc 66 514-519 1944
23 NORTHROP J H *et al* *Crystalline En y es* 2nd edit New York Columbia Un versity Press 1948
24 PAULING L *The Nature of the Che cal Bond* p 64 Ithaca N Y Cornell University Press 1939
25 SMITH E L Aspects of the specificity and mode of act on of some pept d ses in EDSALL J T ed *Enzymes and Enzyme Systems* pp 4 76 Cambridge Mass Harvard University Press 1951
26 STOTZ E *et al* The role of the cytochromes in the act on of in lopl enol oxidase J Biol Chem 124 733 744 1938 The cytochrome C cytochrome oxidase complex *Ib d* 745 754
27 SUMNER J B The isolation and crystallization of the enzyme urease Prel minary paper J Biol Chem 69 435-441 1926
28 UMBREIT W W *et al* *Manometric Techniq es and Tiss e Metabol sm* 2nd edit Minneapol s M nn Burgess Publ sh ng Co 1949
29 WILLIAMS R J *et al* *The B ochemistry of B Vitamins* New York Reinhold Publish ng Corp 1950

CHAPTER 6

Hormones

In addition to enzymes, another group of chemical substances known as *hormones* acts to regulate the reactions continually proceeding in living tissue It is important to differentiate clearly between these two types of chemical mediators

(1) While enzymes are biosynthesized in all cells, hormones are generally produced by specialized glands within the body, such as the pituitary, the thyroid, and the adrenals

(2) Enzymes exert their function in the cells where they are formed or extracellularly after leaving the parent cell Hormones are carried by the bloodstream from the glands where they are formed to the cells within which they act Because the hormone producing glands secrete their products directly into the blood they are often termed the *ductless glands* to differentiate them from such organs as the liver or salivary glands which deliver their secretions by way of a duct into regions other than the blood stream They are also called *endocrine glands*, because they secrete their products into the bloodstream which is within the body, whereas the glands with ducts secrete their products into spaces which are topologically outside the body For the same reason, hormones (derived from a Greek term meaning I arouse to activity') are sometimes called *internal secretions*

(3) Enzymes are invariably proteins whereas hormones although often proteins are not necessarily so

The precise chemical functions of the various hormones have so far proven to be more elusive than those of the various enzymes Whereas the reaction catalyzed by a given enzyme is usually well known, and indeed it is by means of the discovery of the reaction that the enzyme is discovered the manner in which hormones influence reactions is not certainly known for even a single hormone It is logical to suppose, however, that since they are active in exceedingly small concentrations they must act through enzyme systems either as activators or inhibitors or, in the case of the protein hormones conceivably as enzymes in their own right

The effect of hormones on the body is usually marked even startling It is through their presence that body growth is regulated that secondary

sexual characteristics appear at puberty that the body is physiologically mobilized to meet the stresses of fear and anger and so on The hormones of the human body may be divided into two large chemical classes, the *proteins and the steroids plus a few hormones which are neither*

THE PROTEIN HORMONES

The Pituitary Hormones

If there is a master organ in the body one which might poetically be considered the seat of life it is not the liver (as the ancient Greeks thought) or either the heart or brain (as popular modern thought might have it) but the obscure little gland known as the *pituitary* or the *hypophysis* In man it weighs just over half a gram and is situated beneath the brain in the hypophyseal fossa, and is attached to the base of the brain by a thin stalk It is a well hidden, well protected, centrally placed difficult to reach organ as though there had proved to be positive survival value in keeping the pituitary as safe and secure as possible Many of its hormones have as their chief function that of controlling the secretion of hormones produced by the other endocrines, so that the pituitary would seem to be the master mediator of the body The pituitary consists of an anterior lobe, a *pars intermedia* and a posterior lobe each of which produces its own characteristic hormone or hormones, so that functionally the pituitary is really a triple gland The *pars intermedia* has no known significant function in the human All pituitary hormones that have been identified are found to be proteins

Anterior pituitary hormones What has been said about the pituitary gland applies particularly to the anterior lobe from which about 25 different hormonally active fractions have been reported in the literature Of these, six hormones have been well characterized and will be discussed here Two of these may be listed as *gonadotrophic hormones*, that is, hormones which stimulate the growth of the gonads and their production of sex hormones These are *follicle stimulating hormone* (FSH) and *interstitial-cell stimulating hormone* (ICSH) FSH stimulates ovarian follicles and male germ cells while ICSH (also known as luteinizing hormone or LH) similarly stimulates the corpus luteum cells and the interstitial cells of the testes A third hormone affecting tissues associated with the reproductive process is *lactogenic hormone* (also known as prolactin), which initiates and maintains lactation

The other three hormones may be considered metabolic hormones, since they control the rate of metabolism, either indirectly through their stimulation of the thyroid or adrenal cortex in the case of *thyroid-stimulating hormone* (TSH) or *adrenocorticotrophic hormone* (ACTH), respectively, or

through some as yet unknown mechanism in the case of *growth hormone* (also called *somatotrophic hormone*)

These hormones are all produced by the chromophil cells of the anterior lobe The acidophilic cells are associated with the secretion of the growth hormone while the cellular source, acidophil or basophil, of the other hormones is not clearly ascertained The chromophobe or neutrophilic cells of the anterior lobe show no particular evidence of secretory activity

Four of the anterior pituitary hormones have so far been isolated in relatively pure form from the pituitaries of animals These are lactogenic hormone, ICSH, ACTH, and growth hormone Chemically, the anterior pituitary hormones may be divided into glycoproteins (including TSH, FSH and ICSH) and simple proteins (growth hormone, lactogenic hormone and ACTH) Having made this rapid general survey, we may take up the six hormones individually

ICSH. Among the easily available animal sources of pituitary gland tissue, sheep pituitary is the richest in ICSH, possessing some five times the concentration of that in swine pituitary and about twelve times that in beef pituitary (60) Human pituitary is rather low in ICSH (62), being rather comparable to cattle in this respect Pure preparations of ICSH have been prepared both from sheep and from swine, the proteins in each case behaving homogeneously when subjected to electrophoresis, the ultracentrifuge, and solubility tests The hormone preparations from these two animals are quite distinct, differing markedly in molecular weight (sheep ICSH has a 40 000 MW, while that of swine is 100,000) and in isoelectric point (that of sheep ICSH is 4 6 while that of swine is 7 45) Both types of ICSH are glycoproteins containing both mannose and hexosamine Sheep ICSH is about twice as rich as is swine ICSH in both these components, containing 4 5 per cent of mannose and 5 8 per cent of hexosamine as compared to the corresponding figures of 2 8 per cent and 2 2 per cent in swine The activity of ICSH is destroyed when it is treated with ketene (33) and by sufficiently strenuous treatment with cysteine (16) This is interpreted as indicating the essentiality of free amino groups and certain difficultly reducible disulfide groups to the activity of the hormone

FSH. Unlike ICSH follicle stimulating hormone has not yet been prepared in crystal form, so that its chemical properties are not as well known However such information as has been obtained leads to the conclusion that FSH of swine is also a glycoprotein containing both mannose and hexosamine (20) As in the case of ICSH, the pituitaries of swine and sheep are the best source, and the two gonadotrophins can be separated by differential precipitation with ammonium sulfate FSH is soluble in 50 per cent saturated ammonium sulfate solution which is sufficient to precipitate ICSH FSH, in fact, is the only anterior pituitary hormone which is not

precipitated by half saturation with ammonium sulfate FSH is inactivated by cysteine as is ICSH but unlike the latter hormone, is not inactivated by ketene FSH is more resistant than is ICSH to the action of trypsin (9) Human pituitary is rich in FSH (62)

A gonadotrophin of non pitu tary origin appears in the blood and urine of pregnant women Originally it was thought to be identical with the pituitary agents, but it is now known to be formed in the chorion and is for that reason known as *human chorionic gonadotrophin* (HCG) HCG has been crystallized in a form which is electrophoretically homogeneous (10) Earlier analyses of purified preparations 21) indicated that HCG, like the pituitary gonadotrophins was a glycoprotein It differs from ICSH and FSH in that it is richer in carbohydrate si e it contains 10 to 12 per cent hexose and 5 to 6 per cent hexosamine It further differs in that the hexose is not mannose as in the case of the pituitary gonadotrophins but is galactose Its molecular weight is 60 000 to 80 000 and its isoelectric point is about 3 2 The clinical importance of HCG lies in the fact that during the second and third months of pregnancy sufficient hormone passes into the urine for that urine to show pronounced physiological effects upon suitable experimental animals Thus injection of urine of pregnancy into immature female mice or rats will result in the formation of corpora lutea or blood points ' in their ovaries within 96 hours This was the first useful biological test for pregnancy and it and later modifications are termed Aschheim Zondek tests (A Z tests) after the discoverers Later modifications of the A Z test stressed increased quickness of response Male frogs will discharge spermatozoa into the cloaca as a specific response to HCG three hours after an injection of urine of pregnancy

Lactogenic hormone Again in the case of this anterior pituitary hormone, sheep pituitary is a particularly rich source Lactogenic hormone has been prepared in pure crystalline form (61) It is a protein of comparatively low molecular weight, various determinations yielding values between 22 000 and 32 000 the lower value probably being more nearly correct Its isoelectric point is about 5 7 Its solubility properties are rather unusual since it is extremely insoluble (0 01 per cent at 8°C) in distilled water but soluble in methyl or ethyl alcohol in the presence of a small amount of acid As far as is now known, lactogenic hormone is a simple protein and amino acids are its only hydrolysis products

The reaction of lactogenic hormone with iodine or ketene results in the loss of activity indicating that tyrosine and free amino groups are essential to its hormone action The hormone is comparatively resistant to heat Activity of dilute solutions is maintained at a temperature of 60°C over periods of an hour and at 100°C for fifteen minutes The hormone is somewhat more stable in acid than in alkaline solution

Prolactin is concerned with the secretion of the mammary glands, but is *not apparently in* any way concerned with their development, which is under control of ovarian secretion, estrogens and progesterone The stimulus for the release of prolactin seems to be of a duplex nature One of the factors concerned is the fall in estrogen level which occurs at the time of childbirth and another seems to be a reflex stimulation from the act of nursing the baby *The commercial possibilities of prolactin applied to dairy* cattle was thought of quite a while ago It does prolong the period of milk secretion It will not prolong it indefinitely Mammary glands must occasionally still be reconditioned either by a pregnancy or by properly spaced injection of estrogens and progesterone The therapeutic use of prolactin in increasing milk production in the human is by no means uniformly successful We still have to learn more about its necessary conditions of action

TSH. The best practical source of TSH, also known as thyrotrophic *hormone, is the pituitary of cattle or swine It has not yet been prepared* in purified form so that its chemical properties are not very well known It is an even smaller molecule than is lactogenic hormone, its molecular weight being estimated as about 10,000 It is a glycoprotein containing 2 5 per cent glucosamine TSH can definitely be shown to bring about increase in the size of the thyroid, hypertrophy of its cells, and the signs of hyper thyroidism The absence of TSH results in the direct opposite The lowering of the basal metabolic rate (see page 646) of an animal or of a patient *following complete loss of anterior lobe function (by hypophysectomy* or otherwise) is identical numerically with the fall in metabolic rate that takes place following total thyroidectomy

ACTH. The pituitaries of swine and sheep are used as sources for *ACTH* Sheep ACTH has been prepared in pure form (31), the protein proving homogeneous by electrophoresis, ultracentrifuge, and solubility criteria Like lactogenic hormone and TSH, ACTH is a small molecule, its molecular weight being 20 000 It is a simple protein containing no carbohydrate It is higher in sulfur containing amino acids than the other anterior pitui tary hormones which have been characterized in this respect, containing 1 93 per cent methionine and 7 19 per cent cystine (29) Like the other anterior pituitary hormones tested, *it contains no detectable sulfhydryl* groups It is highly soluble in water and fairly stable to heat

The loss of ACTH activity after its reaction with ketene, nitrous acid, formaldehyde, or iodine suggests that free amino groups and tyrosine are among the groups essential for its specific biological action Partial hydrolysis by trypsin or pepsin does not result in loss of activity if not allowed to proceed too far In the case of trypsin, no significant diminution of *ACTH* activity was noted when 18 per cent of the hormone was hydrolyzed as *determined by calculation from* non protein nitrogen analyses When 26

per cent was hydrolyzed, however, all hormonal activity was lost (31) Peptic hydrolysis has even less effect (30) since 50 per cent of the hormone could be hydrolyzed without any loss of activity, and 60 per cent without complete loss Polypeptide fractions have been precipitated by trichloracetic acid from hormone hydrolysates which contain the original activity per unit of nitrogen and there is evidence that some of these active polypeptides may contain as few as eight amino acids Dialysates of such hydrolysates have proved to possess ACTH activity (5), but the chemical nature of the dialyzed material is as yet undetermined

Growth hormone. Of all the hormones of the pituitary, and perhaps of the entire organism growth hormone is the most spectacular in its effects when present in greater or less than the normal amount Growth can be briefly described in chemical terms as the conversion of amino acids into proteins and the orderly storage of such proteins Congenital deficiency of growth hormone results in dwarfism The state of *pituitary infantilism* presumably occurs from the absence of growth and gonadotrophic hormones In some cases, although not in all the pathologist is able to demonstrate atrophy or destruction of the anterior lobe In the classical picture of pituitary infantilism, the body is properly proportioned although small

Conversely, *gigantism* occurs either from hyperplasia or adenoma of the acidophil cells In addition to the oversized body, one usually notes increased basal rate, hyperglycemia, glycosuria, and low sugar tolerance All of these are brought about by the usually associated excess production of other trophic hormones aside from the growth or somatotrophic hormone A late stage in this disease may be a degeneration of the anterior pituitary and the reversal of the metabolic findings Hyperproduction of growth hormone in adults who have previously been normal in this respect stimulates the growth of those parts of the skeleton not yet so mineralized as to be incapable of further extension The main enlargements take place in the feet, hands and lower jaw, and the condition is known as *acromegaly* With the progress of the disease, the chemical findings are frequently reversed, as in gigantism

In a situation like *dystrophia adiposogenitalis*, things cease to be quite so clear cut It is the custom to designate thus as Frohlich's syndrome only when a pituitary tumor can be shown to be present Reputedly, this situation shows the feminine type of adiposity, genital hypoplasia increased sugar tolerance, and hypoglycemia Sometimes, with apparently the same type of obesity and the same type of genital hypoplasia, the pathologist finds nothing at all sometimes he finds a pituitary tumor, and sometimes a hypothalamic tumor The secretion of the gonadotrophins is usually diminished Since the physiology, the pathology, and the physiological pathology of the situation are in such a state of confusion, it is not surprising that

the therapy is equally confused Surgical therapy has been applied The results seem to be rather doubtful Young cases have appeared to do well on the administration of pituitary or chorionic gonadotrophins Older cases have been carried on with some success by the administration of sex hormones Treatment has been directed for the most part at the target organs rather than at what may be the root of the trouble

Growth hormone is one of the four anterior pituitary hormones to be prepared in pure form (32) the protein proving homogeneous by electrophoresis and solubility criteria Its molecular weight is high for an anterior pituitary hormone, about 45 000 Its isoelectric point has been reported as 6 85 It contains no carbohydrate It has been estimated that a molecule of growth hormone contains two residues of tryptophane, four of cystine, nine of methionine, ten of tyrosine and forty of glutamic acid among its more than 350 amino acid residues

The growth hormone is still present in the glands of normal adult animals who have stopped growing So the presence of some sort of inhibitor of the growth hormone which becomes effective at maturity is logical One immediately thinks of the sex hormones as such a possible inhibitor and indeed experimentally, the sex hormones do have an inhibiting effect on the growth hormone of the anterior lobe But this again leads us into a logical trap because castrated animals or castrated humans although a little over growth may be observed certainly do not consistently or for any great period of time show continued increase in size So this is one of the unsolved problems What is it in the normal adult man or animal that at the proper time and in almost all instances holds in check the activity of the growth hormone which can be demonstrated with very little trouble in the adult pituitary gland? The size of almost any animal appears to bear a close relationship to the size of his pituitary gland and whether we deal with species now living or whether we examine skeletons of fossilized vertebrates the relationship between the size of the sella and the size of the animal remains mathematically proportioned (13a)

In pan hypopituitarism or *Simmond s disease* we have the unmistakable signs of extreme and often complete functional failure and often structural absence or structural destruction of the anterior lobe These patients are characterized by their extreme emaciation and extreme or complete regression of the gonads All metabolic activities are depressed and there is hypoglycemia Like rats that have had their anterior lobe removed they develop a profound anemia There are rather few really verified cases where the pathologist has actually found destruction of the anterior lobe Emaciation and loss of practically all body functions certainly can often arise and probably oftener arise from causes other than pan hypopituitarism Here again of course, the basal rate drops very low, down to −50 or less

Posterior pituitary hormones. Extracts of the posterior pituitary lobe have been found to exhibit at least three effects in mammals (a) excitation of uterine muscle contractions (b) increase of the blood pressure, and (c) antidiuresis Whether these effects are due to one, two, or three hormones is not known Two hormones have been named at any rate One, *oxytocin*, is the posterior pituitary principle to which uterine contractions can be attributed while the other *vasopressin*, is held responsible for the increase in blood pressure More conservative workers avoid specific names and dodge the issue of hormone singularity or multiplicity by simply speaking of the pressor oxytocic and antidiuretic effects In any case it seems clear that the hormone or hormones of the posterior pituitary lobe are protein in character

A non crystalline protein has been isolated from the posterior lobe by van Dyke (56) which appeared to be homogeneous by electrophoresis and ultra centrifugation, yet contained the pressor oxytocic, and antidiuretic properties in the same ratio as in the crude extract of the posterior lobe This protein was found to have a molecular weight of 30 000 an isoelectric point at 4 8, and a sulfur content of 4 9 per cent

Those who favor the multiple hormone hypothesis contend that the protein simply has associated with it three polypeptide fragments each displaying a separate property Thus the various properties of posterior pituitary extract were found as long ago as 1899 to be associated with molecules small enough to pass through a dialyzing membrane, and by various methods the molecular weights of the active molecules have been estimated at values varying from 500 to 2 000 This would be equivalent to polypeptides containing from four to sixteen amino acid residues Investigations into the amino acid contents of the oxytocic and pressor fractions showed them to be similar in sulfur content (about 3 per cent), while the oxytocic fraction is somewhat higher in tyrosine and lower in arginine Leucine has been located in the oxytocic fraction while isoleucine occurs in the pressor fraction

Utilizing an active preparation of oxytocic fraction, purified by countercurrent distribution, Pierce and du Vigneaud (43) analyzed the amino acid content by starch chromatography They found it to contain only eight different amino acids in equimolar ratio the eight being leucine, isoleucine, tyrosine proline, glutamic acid aspartic acid, glycine, and cysteine, plus three molecules of ammonia Such an octapeptide would have a minimum molecular weight of 930

Tyrosinase causes complete inactivation, indicating the phenolic OH of the polypeptide to be essential to activity Trypsin rapidly destroys both pressor and oxytocic activities, whereas pepsin has no effect on either of them Cysteine destroys the activity of both pressor and oxytocic fractions, and these can then be reactivated by methylene blue It is interesting that

neither pressor nor oxytocic activity is destroyed by cysteine treatment in the case of van Dyke's active protein molecule containing both activities It may be that the "inactive" portion of the molecule plays an important role in stabilizing the "active" groups Very little is known about the biochemistry of the antidiuretic fraction The mechanism for its release and its effect upon water output is taken up in Chapter 15

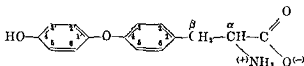
The *pars intermedia* of the pituitary of various species has been shown to contain a hormone whose most pronounced effect is that of causing an expansion of the melanophores of cold blooded animals so that the skin assumes a darker color The hormone the shortest name suggested for it being *intermedin* like those of the posterior pituitary, is a polypeptide of molecular weight about 2 000 It is also similar to the posterior pituitary hormones in that its activity is destroyed by tryptic digestion but not by peptic digestion It differs from them however in that it contains tryptophane which they do not In none of the hormones of the posterior lobe or pars intermedia are any hydrolysis products other than the amino acids or ammonia found

The Thyroid Hormone

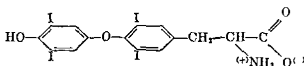
The rate at which oxidative metabolic reactions in general proceed in the body is determined by the endocrine activity of the thyroid gland Following experimental thyroidectomy the rate of oxygen consumption by the resting fasting animal (more succinctly the basal metabolic rate as explained in Chapter 17) is notably decreased below the rate observed in a normal or euthyroid animal The same observation has been repeatedly made in human patients who had been subjected to total or partial thyroidectomy Feeding of thyroid substance to such animals or patients leads to a predictable and measurable increase in basal metabolic rate Feeding of adequate amounts of thyroid substance to normal animals or men will increase the metabolic rate above normal Measurement of oxygen consumption of animal tissues *in vitro* confirms the observations made on the intact animal Tissues of thyroid deficient or *hypothyroid* animals show diminished rates and those from animals rendered *hyperthyroid* by thyroid feeding show increased rates as compared with euthyroid controls Human patients may develop spontaneous hypothyroidism which responds favorably to the feeding of thyroid or they may develop spontaneous hyperthyroidism which may be treated by thyroid blocking drugs or by partial removal or destruction of the thyroid by physical means The exact point in the general oxidative process at which the thyroid hormone exerts its accelerating effect is not known *In vitro* studies have shown that thyroxine at concentrations of 10^{-5} M will inhibit the oxidation of glutamate by rat kidney tissue homogenate (25a) Similar concentrations of thyroxine in

hibited the oxidation of beta hydroxybutyrate by small quantities of kidney homogenate but stimulated oxidation by larger amounts of the homogenate. The relationship of such effects to the general picture of thyroid stimulated metabolism remains speculative.

The thyroid hormone is unusual among the protein hormones in that its active group is definitely known. This active group is the *thyroxine* residue within the polypeptide chain. Because thyroxine itself as well as certain other iodinated amino acids will act to relieve the conditions arising from hypofunction of the thyroid, a certain confusion arises as to exactly what chemical entity may be referred to as the hormone. Salter (51) suggests that "thyroid hormone" be considered a generic term to indicate any substance that will relieve human myxedema when properly administered. Included in such a term would therefore be *thyroglobulin* (the iodinated protein bio



I Thyronine



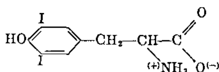
II Thyroxine

synthesized by the thyroid gland), various thyroglobulin degradation products, artificially iodinated proteins, thyroxine and related amino acids, and the thyroxine containing proteins which act as carriers for hormone activity through the bloodstream and into the cell.

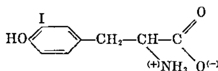
Thyroxine. Most of the amino acids displaying thyroid hormone activity are derivatives of thyronine (formula I), an amino acid, the side chain of which contains two benzene rings connected by an ether linkage. Thyroxine (formula II) itself is 3,5,3′,5′-tetraiodothyronine. L-Thyroxine is by far the most active of the various amino acids with hormonal qualities. D-Thyroxine has only minimal activity. If the two outer iodine atoms in positions 3′ and 5 are removed, the resulting diiodothyronine shows only 4 per cent of the biological activity of thyroxine. If the four iodines are replaced by four bromines, or if the ether oxygen binding the two aromatic rings is replaced by a sulfur, traces of hormone activity (as judged by the ability to relieve human myxedema) remain. If the two benzene rings are each attached directly to the beta carbon, or if three benzene rings (rather

than two) are strung in a row, all activity is lost If the phenol OH is transferred to the 6′ position, 4 per cent of the activity remains, but if it is transferred to the 5′ position while the two neighboring iodine atoms are shifted to 4′ and 6′, all activity is lost

In experiments on tadpoles (whose metamorphosis into frogs can be hastened by thyroid hormone), hormone activity was detected in compounds not containing the thyronine skeleton, as for instance in dibromotyrosine and diiodotyrosine (formula III) The amphibian test is, however, less specific than is that of the relief of human myxedema, and by the former criterion even potassium iodide shows 10 per cent of the hormone activity of thyroxine It seems probable that compounds other than thyroxine show hormone activity in a manner related to the efficiency with which the body can convert them to thyroxine



III Diiodotyrosine



IV Monoiodotyrosine

Thyroglobulin. The thyroid gland contains thyroxine as part of a large protein molecule, thyroglobulin, which apparently serves as a storage place for hormone activity The isoelectric point of the protein is at a pH of about 5 Thyroglobulin contains 0 6 per cent iodine, and since its molecular weight is 700,000, each molecule must have about 32 iodine atoms A technique has been worked out whereby the hydrolyzed thyroid gland of animals who have been treated with radioactive iodine is fractionated by paper chromatography and the iodine fractions visualized by the effect of the radioactive radiation upon a photographic plate (55) In this way, it was determined that 15 per cent of the iodine in rat thyroid existed as monoiodotyrosine (formula IV), and 30 per cent as diiodotyrosine In the chick, the corresponding percentages were 20 and 25 respectively The method was not entirely satisfactory for the determination of thyroxine, but it was estimated that at least 20 per cent and probably much more of the iodine was in that form No 3,5 diiodothyronine (that is, thyroxine minus the two iodines on the outermost ring) was located Neither diiodotyrosine nor thyroxine

exist free in the thyroid to any appreciable extent In addition to these iodinated amino acids, analysis of thyroglobulin indicates the presence in each molecule of 120 cystine residues, 110 tyrosine residues, and 60 residues apiece of tryptophane and methionine Thyroglobulin is a glycoprotein containing some 120 monosaccharide units of which 80 are glucosamine (4)

The non iodinated portions of thyroglobulin are not at all essential to thyroid hormone activity Proteins such as egg albumin, serum albumin, and casein can be treated with iodine and converted into iodinated derivatives which exhibit thyroid hormone activity The reason for this is that the added iodine will first enter the tyrosine residues of the protein molecule to form diiodotyrosine and then by some oxidative process not yet completely elucidated will form thyroxine directly in the polypeptide chain Proteins containing as much as 4 per cent thyroxine have been produced through iodination (48), and it has been shown that diiodotyrosine could to a small extent be converted to thyroxine by alkaline iodination in the test tube

Salter (52) has suggested that the globulin portion of thyroglobulin is synthesized in the thyroid gland independently of iodine metabolism Such an iodine free protein would be a specially designed 'iodine trap' which could rapidly fix iodine absorbed from the foodstuffs Thyroglobulin itself, because of its large molecular weight, is confined within the thyroid cells in which it is formed and can not be expected to be secreted as such into the bloodstream Secretion is preceded by proteolysis (12) into thyroxine containing polypeptides small enough in molecular weight to diffuse readily through the cell walls into the bloodstream In the bloodstream the thyroxine or the polypeptide of which it is part is incorporated into plasma albumin (1) Iodinated plasma albumin then makes its way into the body cells themselves How this is done and what is its function within the cell is not yet known The thyroid gland acts both as a factory and storehouse for the hormone These functions of the thyroid are supervised by the TSH of the anterior pituitary which maintains the gland in an appropriate state of activity

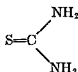
Thyroid-blocking agents. The existence of chemical agents which block the action of the thyroid gland is of interest to the physician in that it offers a method for countering hyperfunction of the gland without resort to surgery Pitt Rivers (46) divides such agents into three groups

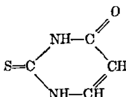
(1) Thiocyanate ion, which by its resemblance in size and charge to iodide ion apparently interferes with the mechanism of iodide collection by the thyroid through competitive inhibition Thiocyanate is metabolized by thyroid tissue and fixed in organic combination, whereas other tissues do not fix thiocyanate sulfur In large doses it will even cause the release of some iodide already concentrated in the thyroid

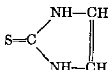
(2) Radioactive iodine I^{130} or I^{131} which by destroying thyroid tissue mechanically reduces the ability of the gland to manufacture hormone

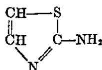
(3) A variety of compounds most of which contain sulfur and which directly or indirectly inhibit hormone synthesis Since such inhibition tends to induce an increase in size of the thyroid as though the gland attempts by this means to restore its hormone production to normal these *thyroid blocking agents* are sometimes referred to as *goitrogens*

The most important goitrogens are derivatives of thiourea (also called thiocarbamide), such as *thiourea* itself (formula V) *2 thiouracil* (formula VI) or *2 mercapto imidazole* (formula VII) An example of a sulfur-containing goitrogen which is not a thiourea derivative is *2 aminothiazole* (formula VIII) Various sulfonamides and even such a non sulfur containing compound as p aminobenzoic acid have been shown to possess goitrogenic activity


V Thiourea


VI 2 Thiouracil


VII 2 Mercapto imidazole


VIII 2 Aminothiazole

The biosynthesis of thyroxine (46) would seem to take place in three stages (a) the conversion of dietary iodide to iodine, (b) the iodination of tyrosine to diiodotyrosine, and (c) the coupling of two diiodotyrosine molecules to form one of thyroxine It is thought that in the case of the thiourea

L L ~f the reducing

reduce iodine

IX) Similar

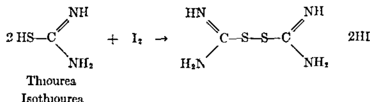
reactions taking place in the body would obviously reduce the amount of *iodine available for the* iodination of tyrosine The goitrogenic activity of such compounds as sulfanilamide and p aminobenzoic acid is more of a puzzle

Goitrogens have been found to occur naturally in certain foods notably rutabaga and turnips The active goitrogenic principle in *turnips has been*

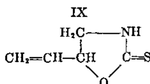
isolated and characterized (19) as a sulfur containing compound which is not however, a thiourea derivative It is L 5 vinyl 2 thio oxazolidone (formula X)

The Pancreatic Hormone

While the pancreas is primarily a ducted gland (second only to the liver in size) discharging a secretion containing a number of digestive enzymes into the duodenum (see Chapter 11), certain cells within it discharge a completely different kind of secretion containing the hormone *insulin* directly into the bloodstream Insulin has the function, discussed in Chapter 12, of promoting the uptake of glucose by cells and the metabolism of glucose within cells Thus the pancreas may also be regarded as having endo



IX



X L-5 Vinyl 2 thio oxazolidone

crinological aspects The insulin-secreting cells occur in little groups which are collectively termed the *islets of Langerhans*, after the discoverer The islets constitute from 0 9 per cent to 2 7 per cent of normal pancreatic tissue and in diabetics the percentage is usually below 0 9 per cent From 250 000 to 2 500 000 individual islets exist in the human pancreas

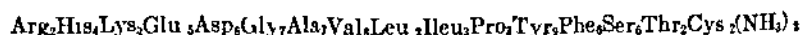
The isoelectric point of insulin is at a pH of 5 3 Its molecular weight as judged from ultracentrifugal sedimentation is 46 000 (38), but the molecule has been found to consist of conglomerates of submolecules of 12 000 molecular weight which are held together by electrovalent bonds The submolecules consist of four polypeptide chains bound together by disulfide linkages Certain facts as to the nature and order of occurrence of the amino acids in the various polypeptide chains are known and have already been described in this book (see page 65) The complete amino acid analysis of insulin utilizing the Brand terminology (3) is shown in formula XI

The activity of insulin is destroyed by alteration of the free amino group ,

as by reaction with formaldehyde, or by the reduction of the disulfide linkages through the action of such chemicals as cysteine thioglycolic acid or leucomethylene blue It is inactivated by pepsin and chymotropsin but is resistant to tryptic digestion

Zinc has been considered to form an integral portion of the insulin molecule although neither the exact manner in which it is bound nor the manner in which it fulfills a function is known (see page 601) Utilizing radioactive zinc the zinc content of crystalline insulin prepared in various ways has been shown to vary from 0 3 per cent to 0 6 per cent depending upon the pH of crystallization (11) although a crystalline insulin containing only 0 15 per cent has been reported (50) The zinc content of human pancreas ranges from 18 5 to 30 4 mgm per kgm of fresh gland Insulin will also crystallize as a salt of other bivalent ions such as those of cobalt or cadmium, and will form a complex with protamine which is of clinical importance since protamine insulin maintains hormonal activity after injection longer than insulin itself (see Chapter 12)

Insulin is comparatively resistant to denaturation by organic solvents dilute acids or by film formation If it is heated in weakly acid solution



$Arg_2His_4Lys_2Glu_5Asp_6Gly_7Ala_7Val_8Leu_2Ileu_3Pro_3Tyr_8Phe_6Ser_6Thr_2Cys_2(NH_3)_8$

XI

inactive fibrils are formed which aggregate into insoluble spherites (59) *This change is reversible and fully active insulin can be regained upon* gentle treatment with alkali

The Parathyroid Hormone

The parathyroid glands are small glands two to four in number usually situated adjacent to the dorsal surface of the thyroids Their total weight is only 100 to 200 mgm The function of the secretion of the parathyroids is that of regulating calcium and phosphorus metabolism (see Chapter 16) The removal of the parathyroids from the body results in hypocalcemic tetany

The nature of parathyroid hormone (PTH) is less well understood than that of any of the hormones thus far discussed Investigation has scarcely reached beyond the point of demonstrating the hormone to be protein in nature That it is a protein is indicated by the fact that hormone activity is lost when active parathyroid extracts are exposed to peptic or tryptic digestion Furthermore such protein denaturants as acid and alkali destroy activity while protein precipitants remove activity from solution The hormone is not a glycoprotein since active extracts react negatively to carbohydrate detecting reagents

No homogeneous preparation of PTH has yet been reported Ross and Wood (49) isolated a hormone preparation which contained at least two

active fractions, separable by ultracentrifugal methods The heavier component, which composed 60 per cent of the total protein weight and 50 per cent of the activity has a molecular weight of from 15 000 to 25 000 Later, a PTH preparation was reported (28) comparable to the Ross Wood hormone in activity per unit nitrogen Ultracentrifugal studies however, showed that there was no fraction equivalent to the Ross Wood heavy component Their equivalent of the Ross Wood light component was, however, itself composed of two fractions as indicated by electrophoretic studies

PTH is less stable in neutral or alkaline media than in slightly acid media Exposure of hormone activity to various reagents indicates that the hormone is stable to reducing agents and unstable to oxidizing agents, that free amino groups are essential to its activity and that disulfide linkages are not present

The Gastrointestinal Hormones

The chemical substances described in Chapter 11 as gastrointestinal hormones are somewhat atypical of hormones as a class, since they are not elaborated by special glands The gastrointestinal mucosa in general seems their region of origin Nevertheless in one important respect they are hormone like in their action since they are secreted directly into the blood by means of which they are carried to the particular region of the digestive tract where they are effective They therefore can be considered internal secretions They are all polypeptide in structure with relatively low molecular weights—secretin, for example, has a molecular weight of about 5,000

If we now pause to view the protein hormones as a group, one important property they seem to hold in common is that of low molecular weight Many seem to be polypeptides rather than proteins in the more common sense of that word and few have molecular weights of more than 20 000 Where larger molecular weights exist, there is the possibility that the isolated molecules represent the "storage" form existing within the hormone synthesizing cells and that the active hormone activity resides in polypeptide components of the protein that fragment off and enter the bloodstream This is certainly the case in thyroid hormone and posterior pituitary hormone, and probably the case in ACTH and parathyroid hormone This small size would seem to be a necessity, of course, if hormones are to be proteins or polypeptides and yet be able to diffuse through the cell walls of the gland and blood vessels into the bloodstream and then from the bloodstream into the receptor cells

THE STEROID HORMONES

Almost all hormones which are not protein in nature are steroids The endocrine glands which secrete steroid hormones are the ovaries, the testes, and the adrenal cortices

The Ovaries

The reproductive function of the human being is not a vital part of the individual's life, regardless of how reluctant the individual may be to have it ignored completely, and is not a matter with which the body is continually preoccupied as it is with respiration and excretion. A woman for instance is capable of bearing young for only about thirty years of a life of which the average expectancy in the United States is nearly seventy. Even within that period a complex four week cycle exists during which she may be fertile for only a day or two.

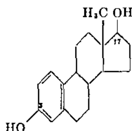
The onset of the menstrual cycle in adolescence, its cessation in middle age, the details of change within each cycle, and the interruption of the cycle by the advent of pregnancy and parturition all involve profound physiological and even anatomical changes. To induce such changes in individual parts of the body without at any time allowing the organism as a whole to have its metabolic machinery seriously interfered with is an important function of those chemical messengers and regulators, the hormones. The ovaries and testes in addition to their reproductive function also elaborate a variety of important hormones.

The ovarian hormones fall into two classes, the estrogens and progesterone. The *estrogens* are biosynthesized by the maturing ovarian follicle. Some half million exist in the ovarian cortex, but few of these (ideally one every lunar month) mature to produce an ovum. *Progesterone* is formed in the corpus luteum cells which arise from the hypertrophy of the matured follicle. The production of these ovarian hormones is regulated by the gonadotrophins of the anterior pituitary.

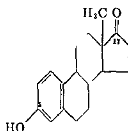
Estrogens. Three closely related steroids with estrogenic activity are formed in the human ovary, differing only in the number of hydroxy or keto groups present on the steroid nucleus (CPP). They are *estradiol* (formula XII), *estrone* (formula XIII), and *estriol* (formula XIV). If we compare the three we see that estradiol and estrone differ only in that the 17 hydroxyl of the former is oxidized to a 17 ketone in the latter. Estriol differs from estradiol in the possession of a third hydroxyl group on carbon 16. As a group, the three estrogens differ from most other steroids in that ring A is fully aromatic so that these steroids lack the usual angular methyl on carbon 10. This aromaticity is carried even further in *equilin* (formula XV) and *equilenin* (formula XVI), related estrogens which occur in the horse but not in the human. Estrone and estradiol occur in all species studied but estriol seems characteristic of the human (41).

The estrogens control the development of secondary sexual characteristics in the female and are responsible as well for the maintenance of the menstrual cycle. After ovariectomy the cycle is interrupted and can be restored by injections of estrogens, which will also reverse the consequent

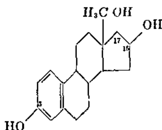
atrophy of organs essential or accessory to reproduction—as the uterus vagina, oviducts, and breasts Circulating estrogens are also believed to be responsible for rebuilding the endometrium after menstruation Estradiol is the most active of the estrogens with estrone and estriol following in that order



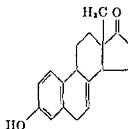
XII Estradiol



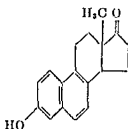
XIII Estrone



XIV Estriol



XV Equilin



XVI Equilenin

The estrogens are not formed in the ovaries alone Estrogen excretion rises considerably during the course of pregnancy and drops very rapidly after parturition, indicating that the placenta also may be involved with estrogen synthesis Placental tissue contains a high concentration of estrogens In addition, estrogens have been isolated from the other two organs known to synthesize steroid hormones, the adrenal cortex and the testes The last may seem somewhat unexpected For some reason the horse testis

is an extremely active estrogen producer and the richest known natural source of estrogens is the urine of stallions (27)

The urine of pregnant women is rich in estrogen, its excretion rising to 8,000 mouse units per day just before parturition (42) Twelve days after parturition urinary excretion has dropped to virtually indetectable values and remains so for two weeks In non pregnant women and in men urinary estrogen values are low (see table 10)

Urinary estrogens occur as conjugates, either of glucuronic acid as in the case of estriol or of sulfuric acid as in the case of estrone Estradiol, the most active of these hormones, is apparently the parent compound of the human estrogens It is in equilibrium with estrone, to which it may be reversibly oxidized in the body Estrone may be hydrated to estriol in an

TABLE 10

Estrogen values in urine of men and non pregnant women (8)

In International Units/24 hours

	RANGE	AVERAGE
Female (days of menstrual cycle)		
7	65–160	110
14	160–660	330
21	160–660	500
28	30–110	65
Menopausal woman	10– 65	under 65
Male	25–100	50

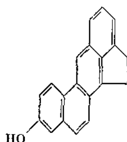
One International Unit (I U) is equivalent in estrogenic activity to that of 0 1 μg of crystalline estrone

irreversible fashion This has been determined by feeding individual estrogens and then analyzing the urine for excreted products When estrone or estradiol is fed, all three forms may be found in the urine, but when estriol is fed, only it is excreted However, some 80 to 90 per cent of ingested estrogen is converted to non hormonal metabolic products and the state of knowledge of estrogen metabolism is anything but satisfactory

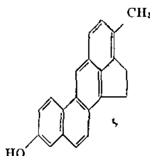
Perhaps the most interesting possibility concerning these metabolic products of the estrogens is that polycyclic hydrocarbon carcinogens may be biosynthesized Fieser has suggested (15) that equilenin (or estrone after suitable dehydrogenation) might condense with pyruvic acid and form, after further dehydrogenation, 3 hydroxycholanthrene 3 Hydroxycholanthrene (formula XVII) has not been prepared synthetically so that its carcinogenicity is not known, but 3 hydroxy 20 methylcholanthrene (formula XVIII) has been synthesized and it is not carcinogenic, the 3 hydroxy apparently neutralizing the highly potent carcinogenicity of 20 methyl

cholanthrene itself This is a point against the theory of the conversion of estrogen into carcinogen More recently, however 3-desoxyequilenin (formula XIX) has been isolated from the urine of pregnant mares and this upon condensing with pyruvic acid in the aforementioned manner would produce cholanthrene which is known to be a potent carcinogen

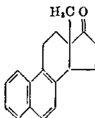
Our present knowledge of the biosynthesis of estrogens is even more fragmentary than that of their catabolism Pro estrogens are claimed to exist in the ovaries of infantile female rats during the eighteen hour period



XVII 3 Hydroxy-cholanthrene



XVIII 3 Hydroxy 20 methylcholanthrene



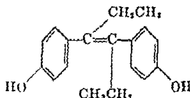
XIX 3 Desoxyequilenin

following the injection of chorionic gonadotrophin (63), but the exact chemical structure of such pro-estrogen is not known

Considerable evidence exists to the effect that liver is a major site of estrogen catabolism Estrogens have been found to lose activity when incubated with liver slices or certain liver extracts (14) The inactivation can be quite rapid and is attributed by some to the presence of an estrinase in the liver cell *In vivo* studies confirm this liver role Animals with damaged or partially removed livers proved to be more sensitive to estrogen administration indicating a slowdown in the rate of estrogen inactivation (54) In male patients with liver disease, increased concentrations of estrogens probably are present in blood and tissues, since such patients have shown atrophy of testicles and enlargement of breasts

The end products of estrogen inactivation are not known The reaction catalyzed by the postulated estrinase must be more than a detoxication by such usual mechanisms as glucuronide formation since the estrogen glucuronides display feeble estrogenic activity whereas the liver inactivated products are completely inactive It may be that the *in vivo* inactivation of estrogens by the liver is far from as rapid as *in vitro* studies have indicated and that it may not keep up with the supply reaching it via the blood This is indicated by the fact that the bile in dogs and in humans has been found to be high in estrogens in the case of both endogenous secretion and exogenous administration (7) Excretion by the bile would thus be *another mechanism whereby surplus estrogens are disposed of by the liver*

There are several synthetic estrogens The most important of these is *diethylstilbestrol* (formula XX) also known simply as stilbestrol It is of great importance clinically since it *is not only cheaper than the natural* estrogens but is more potent than is estrone



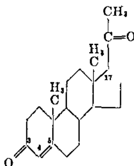
XX Diethylstilbestrol

Progesterone Progesterone is synthesized by the corpus luteum which is formed from the ovarian follicle after ovulation The secretion continues during the second half of the menstrual cycle stopping a few days before the onset of menstruation Its effects thus follow *the earlier action of the* estrogens Progesterone causes an increase in vascularity and secretory activity of the endometrium preparing it for the implantation of the fertilized ovum *Uterine motility is inhibited by progesterone* Menstruation follows cessation of progesterone secretion but should pregnancy intervene progesterone continues to be elaborated by the persistent corpus *luteum* under the influence of the pituitary gonadotrophins and the vascularity of the endometrium continues to increase In the later stages of pregnancy the placenta becomes an important site *for the formation of progesterone* As in the case of the estrogens small quantities of progesterone are formed by the adrenal cortex and probably by the testes as well

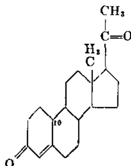
Progesterone like the other ovarian hormones is a steroid It differs from the estrogens in the following respects (a) ring A is not aromatic but has a single double bond between carbons 4 and 5 *This does not involve* carbon 10 and progesterone (formula XXI) has an angular methyl at that position—unlike the estrogens (b) The oxygen at position 3 is *ketonic*

rather than phenolic And (c) the side chain at position 17 is $-COCH_3$, rather than $-OH$ or $=O$

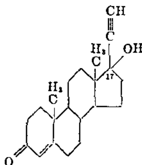
Synthetic compounds similar to progesterone also possess hormone activity, as, for instance, those in which the C 17 side chain is $-COCH_2CH_3$ or $-CHO$, or where the angular methyl at C 10 is absent This last compound, *10 norprogesterone* (formula XXII), is as active as and possibly more



XXI Progesterone



XXII 10 Norprogesterone



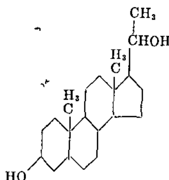
XXIII 17-Ethinyltestosterone

active than progesterone itself Still another compound, *17 ethinyltestosterone* (formula XXIII), which differs from progesterone in that there are two substituents on the C 17, a hydroxy group and a $-C\equiv CH$ (ethinyl) group, not only displays about one third the progestational activity of progesterone but, in the rabbit at least, is effective when administered orally, which progesterone itself is not Other substances showing progestational activity are androgens such as testosterone and synthetic methyl testosterone In all cases the keto group at C-3 and the double bond between C-4 and C 5 seem to be essential to activity

Progesterone is definitely known to undergo reduction in the body to

pregnanediol (formula XXIV), which may be detected in the urine as a glucuronide in which the glucuronic acid residue is attached to the oxygen on C-3 Pregnanediol differs from progesterone in that both keto groups are reduced to the alcohol and that the double bond of ring A is hydrogenated Although during pregnancy many compounds chemically related to pregnanediol are normally excreted in the urine, only pregnanediol itself was detected in the urine of non pregnant women after the oral administration of progesterone (57) It is supposed from this that metabolism of progesterone differs in pregnant and *non-pregnant women or else that in pregnant* women compounds related to but not identical with progesterone are formed

A woman in the eighth month of pregnancy, fed cholesterol tagged with deuterium, was found to excrete in her urine pregnanediol with a sufficient



XXIV Pregnanediol

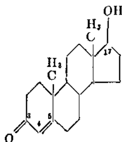
deuterium content to indicate that one half to two thirds of it arose by the degradation of cholesterol (2) Presumably, the conversion of cholesterol to pregnanediol went by way of progesterone, and this is the most direct evidence yet available to connect cholesterol and the ovarian hormones Earlier work had indicated that cholesterol, after incubation with liver slices, yielded products possessing estrogenic activity, but this line of attack has not been followed up As in the case of the estrogens, the chief site of progesterone catabolism is the liver

The Testes

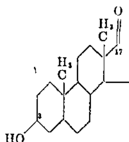
Androgens. The androgens are synthesized primarily in the interstitial tissue of the testes, and their manufacture is under the control of the pituitary gonadotrophins The androgens control the development of the masculine secondary sexual characteristics, such as hair distribution and change in voice, as do the estrogens in analogous fashion for the female Furthermore they maintain the normal functional condition of the accessory sex

organs of the male, especially the prostate and the seminal vesicles To gether, the testicular and ovarian hormones are referred to as the *sex hor mones* or, more logically but more rarely, the *sexogens*

While the androgens are synthesized primarily in the testes androgenic material is normally found also in the secretions of the ovary and the ad renal cortex The androgen characteristic of the testes is *testosterone* (for mula XXV) Two androgens of considerably lower potency had been iso lated from male human urine and characterized before testosterone was isolated from bull testes These are *androsterone* (formula XXVI) and *de hydroisoandrosterone (formula XXVII) Androsterone is only one sixth as* active as testosterone It will be noted that testosterone seems to be estrone in reverse as far as the oxygen containing groups on the CPP nucleus are concerned While estrone is a 3 hydroxy, 17 keto compound testosterone is a 3 keto 17 hydroxy compound An additional difference is of course



XXV Testosterone

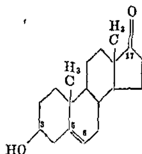


XXVI Androsterone

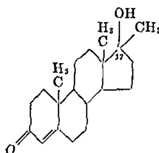
that ring A of testosterone is not aromatic as is that of estrone and the C 10 angular methyl is present The ring system of testosterone is exactly that of progesterone and the only difference between these two compounds is the nature of the grouping on C 17 a hydroxyl in the former and a meth ylketo in the latter It is presumably this similarity in the ring system that enables testosterone and *17 methyltestosterone* (formula XXVIII) to display progestational activity

Androsterone like estrone is a 3 hydroxy, 17 keto compound but it differs from estrone in being fully saturated and possessing the C-10 angular methyl Dehydroisoandrosterone differs from androsterone in possessing one double bond between carbons 5 and 6 and having its C-3 hydroxyl group in the beta configuration rather than the alpha configuration of an drosterone A number of other androgens have been isolated from urine testes and adrenal cortex displaying varying combinations of keto and hydroxy groups with saturated rings or with one double bond appearing in some one of the four rings (and in one case in two of the four rings), but none of these are particularly significant

Virtually nothing is yet known about the biosynthesis of androgens Knowledge of their catabolism is in a somewhat better state The administration of testosterone either orally or intramuscularly is followed by the excretion of androgens and other 17 ketosteroids in the urine The urinary androgens are in a water soluble, inactive form, having been conjugated with sulfuric acid Dehydroisoandrosterone sulfate and androsterone sulfate have been isolated from the urine (39, 58a) The most common additional 17-ketosteroid in human urine is etiocholanol-3(alpha) one 17, which



XXVII Dehydroisoandrosterone



XXVIII 17 Methyltestosterone

differs from androsterone in that rings A and B are *cis* in the former and *trans* in the latter As in the case of the ovarian hormones, testosterone is inactivated in the liver

The functions of spermatogenesis by the seminiferous tubules and of androgen production by the interstitial cells of Leydig are closely related but may vary independently of each other They are stimulated by different pituitary hormones, FSH for spermatogenesis, and ICSH for androgen production Administration of large amounts of androgen (e g , 50 mgm. testosterone daily) will inhibit spermatogenesis in man, probably by inhibition of formation of pituitary FSH, and may even produce histologically demonstrable degeneration of the seminiferous tubules Following inhibition by

testosterone a gradual recovery of spermatogenic tissue may occur, with eventual increase of spermatozoa counts above the pre treatment level

17-Ketosteroids. The 17 ketosteroids are properly speaking, all compounds containing the CPP nucleus with an oxygen doubly bound to C-17 In this sense, estrone androsterone, and dehydroisoandrosterone, among *the natural sexogens may be considered 17 ketosteroids The term is usu*ally applied, however, only to the neutral 17 ketosteroids which remain in the urine extract after treatment with aqueous alkali to remove phenolic steroids such as estrone The neutral 17 ketosteroids arise chiefly from substances originally secreted by the testes or the adrenal cortex With the exception of androsterone and dehydroisoandrosterone, these display little or no sexogen activity They are however of considerable interest to the

TABLE 11

Androgen and 17 ketosteroid content in the urine of normal males and females (13)

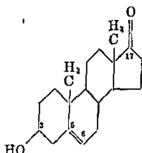
AGE GROUPS	MALES		FEMALES	
	Androgens	17 Ketosteroids	Androgens	17 Ketosteroids
years	*I U /day*	*mgm /day*	*I U /day*	*mgm /day*
under 8	less than 1	2 8	1– 2	1 4
8–12	4 8	4– 8	5– 7	5– 7
12 15	9– 11	8–11	8–18	6–12
15–18	22 25	—	—	—
20–40	20–100	10–20	10–80	5–12
over 50	5– 20	4–10	9–11	—

One International Unit (I U) is equivalent in androgenic activity to that of 0 1 mg androsterone

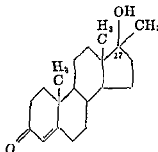
physician because of the diagnostic applications of urinary 17 ketosteroid values These values are determined by photometric methods The most commonly used methods take advantage of the fact that 17 ketosteroids will react with *m*-dinitrobenzene in an alkaline alcoholic medium to produce strongly colored complexes (6 23) More recently, Pincus has described a method (44) which involves the reaction of neutral ketosteroids with concentrated antimony trichloride in acid solution Androsterone and its isomers produce an intense blue color, whereas the 20 ketosteroids and 3 ketosteroids give yellowish or colorless reaction products

The androgen and 17 ketosteroid content of the urine at various age levels is remarkably uninfluenced by sex (see table 11) The fact that the picture in young girls is similar to that in young boys may be explained partly by the fact that the adrenal cortex contributes 17 ketosteroids to the urine in both sexes What parallels in the girl, however, the increasing androgen and androgen-descended 17 ketosteroid in boys' urine as puberty

Virtually nothing is yet known about the biosynthesis of androgens Knowledge of their catabolism is in a somewhat better state The administration of testosterone either orally or intramuscularly is followed by the excretion of androgens and other 17 ketosteroids in the urine The urinary androgens are in a water soluble, inactive form, having been conjugated with sulfuric acid Dehydroisoandrosterone sulfate and androsterone sulfate have been isolated from the urine (39, 58a) The most common additional 17 ketosteroid in human urine is etiocholanol-3(alpha) one-17, which



XXVII Dehydroisoandrosterone

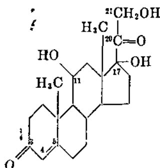


XXVIII 17 Methyltestosterone

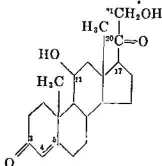
differs from androsterone in that rings A and B are *cis* in the former and *trans* in the latter As in the case of the ovarian hormones, testosterone is inactivated in the liver

The functions of spermatogenesis by the seminiferous tubules and of androgen production by the interstitial cells of Leydig are closely related, but may vary independently of each other They are stimulated by different pituitary hormones, FSH for spermatogenesis, and ICSH for androgen production Administration of large amounts of androgen (e g , 50 mgm. testosterone daily) will inhibit spermatogenesis in man, probably by inhibition of formation of pituitary FSH, and may even produce histologically demonstrable degeneration of the seminiferous tubules Following inhibition by

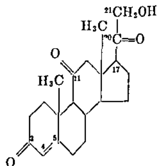
oxygens are at carbons 3, 11, 20, and 21 The ninth four-oxygen corticoid is not yet completely characterized chemically Of these corticoids, three are of major importance Two belong to the 17-desoxy group which is also known, after its most important member, as the corticosterone group The two are *corticosterone* (formula XXXII) itself, and *11-dehydrocorticosterone* (formula XXXIII) The third belongs to the 11-desoxy group and is *11-desoxy 17 hydroxycorticosterone* (formula XXXIV)



XXXI 17-Hydroxycorticosterone



XXXII Corticosterone



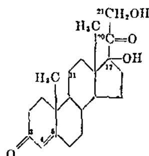
XXXIII 11-Dehydrocorticosterone

Five corticoids possess three oxygens apiece In four of these, the oxygens are situated at carbons 3, 17, and 20 In the fifth, *11-desoxycorticosterone (formula XXXV), the most important* of the group, the oxygens are on carbons 3, 20, and 21

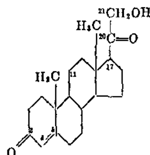
Finally, the last two of the 21-carbon corticoids contain only two oxygens apiece, on carbons 3 and 20 One of these is progesterone

There remain four corticoids with less than 21 carbons in the molecule Three possess 19 carbons, having no 2 carbon side chain on carbon 17 At least two of these show androgenic properties, *androstene-3,17-dione* (formula XXXVI) being as effective as androsterone in this respect, and

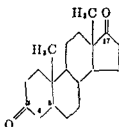
adrenosterone (formula XXXVII) one fifth as effective Lastly, we have estrone which because of the phenolic character of ring A and the consequent absence of one angular methyl has only 18 carbons in its molecule



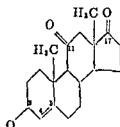
XXXIV 11 Desoxy 17 hydroxy corticosterone



XXXV 11 Desoxy corticosterone



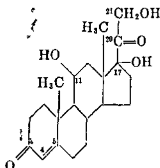
XXXVI Androstene-3 17 dione



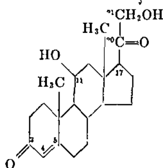
XXXVII Adrenosterone

In addition to all these compounds there remains a so-called "amorphous fraction", in the mother liquor when all known steroids have been crystallized out of solution This amorphous fraction is highly potent with respect to life maintenance in adrenalectomized animals, being 50 per cent as

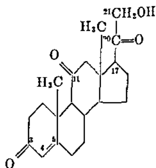
oxygens are at carbons 3, 11, 20, and 21 The ninth four oxygen corticoid is not yet completely characterized chemically Of these corticoids, three are of major importance Two belong to the 17-desoxy group which is also known, after its most important member, as the corticosterone group The two are *corticosterone* (formula XXXII) itself, and *11-dehydrocorticosterone* (formula XXXIII) The third belongs to the 11-desoxy group and is *11-desoxy 17 hydroxycorticosterone* (formula XXXIV)



XXXI 17-Hydroxycorticosterone



XXXII Corticosterone



XXXIII 11 Dehydrocorticosterone

Five corticoids possess three oxygens apiece In four of these, the oxygens are situated at carbons 3, 17, and 20 In the fifth, *11-desoxycorticosterone* (formula XXXV), the most important of the group, the oxygens are on carbons 3, 20, and 21

Finally, the last two of the 21-carbon corticoids contain only two oxygens apiece, on carbons 3 and 20 One of these is progesterone

There remain four corticoids with less than 21 carbons in the molecule Three possess 19 carbons, having no 2 carbon side chain on carbon 17 At least two of these show androgenic properties, *androstene-3,17-dione* (formula XXXVI) being as effective as androsterone in this respect, and

coneogenesis Certain side effects accompany the metabolic function One of these is the increased excretion of uric acid so that the urinary uric acid creatinine ratio may serve as an index of adrenocortical activity The presence of the adrenal cortex is necessary to initiate the eosinopenia which follows stress in rats Once started the drop in eosinophils continues even though adrenalectomy is performed within 10 minutes after the induction of stress by adrenalin injection The decrease of circulating eosinophils seems to be well established and is used as an indicator of hormone output by the adrenal In mice the drop in eosinophils can be explained by accumulation in the spleen

Cortisone exemplifies the metabolic effect of adrenocortical secretion It is 17 hydroxy 11-dehydrocorticosterone (Kendall s Compound E) It shows very slight electrolyte effect At first the compound was available only by isolation from animal adrenals The supply has been greatly, but still not adequately enhanced by the development of synthetic procedures Hench of the Mayo Clinic (22) first proposed its use in diseases not obviously connected with adrenocortical deficiency Its remarkable ameliorating effect in some varieties of arthritis has been amply described in both the medical and the lay press

The diabetes produced in experimental animals by pancreatectomy is mitigated by adrenalectomy and aggravated by metabolically active adrenal steroids This effect is explained in part by increased gluconeogenesis from protein which is characteristic of the action of such steroids Glycosuria can however be produced by administering corticosterone cortisone or ACTH to normal rats who are forcibly overnourished The nitrogen excreted by such rats is not adequate to account for the production of the observed amounts of glucose excreted solely on the basis of gluconeogenesis from protein Other possibilities are gluconeogenesis from fat and decreased ability to utilize carbohydrate Glycosuria can be produced in normal human subjects by injection of ACTH and has been produced in patients under ACTH therapy

Cortisone increases the concentration of plasma amino acids in eviscerated rats maintained by intravenous saline glucose and insulin and increases the concentration of blood cholesterol in human patients Cortisone accelerates the progress of experimental poliomyelitis infection in mice (53) ACTH does not produce this accelerating effect possibly because it simultaneously promotes the output of an oppositely acting steroid The prolonged use of cortisone (or ACTH) in human patients with active or even latent tuberculosis is reported to have accelerated the progress of the disease (18) Similar effects on laboratory animals had earlier been reported

In contrast cortisone inhibits many of the manifestations of inflammation such as the invasion of polymorphonuclears The effect of cortisone

active as the original material in this respect (24) The amorphous fraction is low however in those steroids specifically concerned with carbohydrate metabolism The chemical nature of the steroids of the amorphous fraction is not yet known but they are thought to belong to the 21 carbon o-oxygen series

Chemical structure and physiological action The activity of corticoids may be tested in a number of ways such as (a) by their effect on the length of survival of an adrenalectomized animal (b) by their influence on sodium ion retention (c) by their effect in preventing insulin convulsions in intact rats and (d) by their influence on the rate of glycogen deposition in adrenalectomized rats It has been found that the position of individual corticoids on the scale of activity depends upon the test used The types of corticoid activity have been found associated with chemical structure

The physiologically measurable effects of the adrenocortical steroid hormones can be separated into two major categories One of these may be briefly designated the *electrolyte effect* since it involves water and ionized salts In human patients and in most laboratory animals deficiency of the adrenal cortex is characterized by increased urinary output of Na^+ Cl HCO_3 and water To a lesser degree K^+ and HPO_4^- are retained To phrase it in the opposite manner a function of some of the adrenal hormones is to promote the renal tubular absorption of Na^+ Cl HCO_3 and water and the excretion of K^+ and HPO_4^-

The compound 11 desoxycorticosterone best exemplifies this action This is the familiar synthetic marketed as desoxycorticosterone acetate or DOCA and used in the therapy of Addison s disease It is not a major component of the steroids obtained directly by fractionation of the adrenal cortex although it is present there 11 Dehydrocorticosterone and corticosterone (Compound A and Compound B) show a similar action to a lesser degree Although the effect upon water and salts is mediated chiefly by the kidney other related activities have been demonstrated which appear to be entirely extra renal Synthetic DOCA inhibits the output of sodium in sweat and increases diffusion of Na^+ and Cl into the peritoneal cavity of rabbits

When a portion of the liver is removed surgically from a rat its rate of regeneration is slower in an adrenalectomized than in a normal animal DOCA is the most potent corticoid in this particular function with 11 dehydrocorticosterone a close second definitely ahead of cortisone

The second major activity of adrenocortical steroid hormones may be called the *metabolic effect* In a very broad sort of way this may be summarized as the maintenance of glycogen stores and the blood sugar level promotion of the intestinal absorption of fat and the acceleration of glu

coneogenesis Certain side effects accompany the metabolic function One of these is the increased excretion of uric acid so that the urinary uric acid-creatinine ratio may serve as an index of adrenocortical activity The presence of the adrenal cortex is necessary to initiate the eosinopenia which follows stress in rats Once started the drop in eosinophils continues even though adrenalectomy is performed within 10 minutes after the induction of stress by adrenalin injection The decrease of circulating eosinophils seems to be well established and is used as an indicator of hormone output by the adrenal In mice the drop in eosinophils can be explained by accumulation in the spleen

Cortisone exemplifies the metabolic effect of adrenocortical secretion It is 17 hydroxy 11-dehydrocorticosterone (Kendall s Compound E) It shows very slight electrolyte effect At first the compound was available only by isolation from animal adrenals The supply has been greatly, but still not adequately, enhanced by the development of synthetic procedures Hench of the Mayo Clinic (22) first proposed its use in diseases not obviously connected with adrenocortical deficiency Its remarkable ameliorating effect in some varieties of arthritis has been amply described in both the medical and the lay press

The diabetes produced in experimental animals by pancreatectomy is mitigated by adrenalectomy and aggravated by metabolically active adrenal steroids This effect is explained in part by increased gluconeogenesis from protein which is characteristic of the action of such steroids Glycosuria can however be produced by administering corticosterone cortisone or ACTH to normal rats who are forcibly overnourished The nitrogen excreted by such rats is not adequate to account for the production of the observed amounts of glucose excreted solely on the basis of gluconeogenesis from protein Other possibilities are gluconeogenesis from fat and decreased ability to utilize carbohydrate Glycosuria can be produced in normal human subjects by injection of ACTH and has been produced in patients under ACTH therapy

Cortisone increases the concentration of plasma amino acids in eviscerated rats maintained by intravenous saline glucose and insulin and increases the concentration of blood cholesterol in human patients Cortisone accelerates the progress of experimental poliomyelitis infection in mice (43) ACTH does not produce this accelerating effect, possibly because it simultaneously promotes the output of an oppositely acting steroid The prolonged use of cortisone (or ACTH) in human patients with active or even latent tuberculosis is reported to have accelerated the progress of the disease (18) Similar effects on laboratory animals had earlier been reported

In contrast cortisone inhibits many of the manifestations of inflammation such as the invasion of polymorphonuclears The effect of cortisone

Cortisone alone does not provide adequate therapy for Addison's disease but is a part of an optimal regime which should also include Na^+ and DOCA. Evidence is at hand (40) that cortisone reduces the rate of growth of lymphoid tumors, clinical evaluation of this evidence is in progress at several medical centers

Emphasis on cortisone and related metabolic hormones has somewhat overshadowed the long recognized androgenic and estrogenic effects of adrenocortical hormones. These effects are most conspicuous in tumors of the adrenal cortex, which have been reported to have masculinizing or feminizing effects. Substances with androgenic and other substances with estrogenic effects have been identified as components of the adrenal corticoids. Associated with the androgenic substance or substances is the related property of nitrogen storage by protein synthesis.

Returning to the chemical structure of the corticoids, we see that as far as rings A and B are concerned the double bond between carbons 4 and 5 and the keto group on carbon 3 are essential to a high degree of corticoid activity of any type. All six of the highly active 21-carbon corticoids possess this chemical grouping. Such a grouping is found also in progesterone, testosterone, and the androgenic corticoids.

If ring C is considered, then it is found that only those compounds bearing an alcoholic or ketonic oxygen at C 11 exhibit any effect on carbohydrate metabolism. These are therefore called the *glycocorticoids*. The 11-oxy compounds cause deposition of glycogen in the liver of the adrenalectomized animal, prevent insulin convulsions in intact rats, and cause increased glycosuria in partially depancreatized and in adrenalectomized-depancreatized rats. The 11-desoxy compounds which are less effective in these respects than the 11-oxy compounds are more effective with respect to the life maintenance of adrenalectomized animals and in the recovery of fatigued muscle in adrenalectomized rats (the Everse-de Fremery test). 11 Desoxy corticoids also increase sodium retention, and are referred to as *mineralocorticoids*.

With respect to the carbon 17,2-carbon side chain, the $—COCH_2OH$ is essential for high activity and occurs in all six of the most active corticoids. The one test in which corticoids not containing this side chain show considerable activity is in life maintenance of adrenalectomized animals. Progesterone, for instance, which has the $—COCH_3$ side chain is fairly effective in this respect, although it lacks other types of corticoid activity. The presence of a hydroxy group on C-17, as is the case in three of the six chief corticoids, gives rise to a marked diminution of potency in the life maintenance and Everse-de Fremery tests but enhances activity with respect to carbohydrate metabolism.

One non-steroid which exhibits a small amount of corticoid potency is,

on concentration of circulating antibody has not been settled reports in the literature are conflicting Since inflammation of either traumatic or allergic origin is equally inhibited it has been deemed unlikely that cortisone acts by blocking the antigen antibody reaction Cortisone has been used locally for inhibiting *inflammatory changes as for example by* subconjunctival injection in iritis A more familiar application is the systemic use of ACTH in the treatment of extensive thermal burns A related specific action *is the inhibition of hyaluronidase*

In spite of the fact that cortisone diminishes or abolishes the manifestations of hypersensitivity local passive transfer of hypersensitivity by serum *can be accomplished from cortisone treated donors and to cortisone treated* recipients as demonstrated by positive Prausnitz Kustner reactions and with no diminution in intensity resulting from the use of cortisone The *question has been raised as to* whether by paralyzing *natural defense* mechanisms cortisone may not accelerate the spread of infections A ... mumps virus in develop

... DOCA on plasma potassium Cortisone produces an elevation of plasma K^+ If both drugs are given together the DOCA *effect* depression of plasma K^+ *predominates* These observations were made in rats Cortisone does not however antagonize the cardiovascular renal effects of DOCA

A similar antagonistic effect is shown in the elevation of the electro shock seizure threshold in rats which is elevated by DOCA and restored to normal by ACTH or extracts of adrenal cortex This and other evidence indicates that DOCA inhibits the ACTH production of the anterior pituitary

Definite and verifiable information is still scant concerning the mode of action of cortisone (and of ACTH which presumably *acts chiefly* by increasing the output of cortisone and related metabolic hormones) in relation to the therapy of the collagen diseases The group of collagen diseases includes rheumatic fever rheumatoid arthritis *scleroderma* periarteritis nodosa disseminated lupus erythematosus dermatomyositis and psoriasis This group of diseases is characterized by alterations of collagen connective tiss ... plasma protein ... in the therapy ... nnally effective ... tient s tissues to repair the damaged mesenchymal system Malkiel (50 ... however reports ACTH and cortisone to be without any apparent effect on the end results of histamine shock *or of passive or active* anaphylaxis in guinea pigs

Cortisone alone does not provide adequate therapy for Addison's disease, but is a part of an optimal regime which should also include Na^+ and DOCA. Evidence is at hand (40) that cortisone reduces the rate of growth of lymphoid tumors, clinical evaluation of this evidence is in progress at several medical centers.

Emphasis on cortisone and related metabolic hormones has somewhat overshadowed the long recognized androgenic and estrogenic effects of adrenocortical hormones. These effects are most conspicuous in tumors of the adrenal cortex, which have been reported to have masculinizing or feminizing effects. Substances with androgenic and other substances with estrogenic effects have been identified as components of the adrenal corticoids. Associated with the androgenic substance or substances is the related property of nitrogen storage by protein synthesis.

Returning to the chemical structure of the corticoids, we see that as far as rings A and B are concerned the double bond between carbons 4 and 5 and the keto group on carbon 3 are essential to a high degree of corticoid activity of any type. All six of the highly active 21 carbon corticoids possess this chemical grouping. Such a grouping is found also in progesterone, testosterone, and the androgenic corticoids.

If ring C is considered, then it is found that only those compounds bearing an alcoholic or ketonic oxygen at C 11 exhibit any effect on carbohydrate metabolism. These are therefore called the *glycocorticoids*. The 11 oxy compounds cause deposition of glycogen in the liver of the adrenalectomized animal, prevent insulin convulsions in intact rats, and cause increased glycosuria in partially depancreatized and in adrenalectomized depancreatized rats. The 11-desoxy compounds which are less effective in these respects than the 11 oxy compounds are more effective with respect to the life maintenance of adrenalectomized animals and in the recovery of fatigued muscle in adrenalectomized rats (the Everse-de Fremery test). 11 Desoxy corticoids also increase sodium retention, and are referred to as *mineralocorticoids*.

With respect to the carbon 17,2 carbon side chain, the $-COCH_2OH$ is essential for high activity and occurs in all six of the most active corticoids. The one test in which corticoids not containing this side chain show considerable activity is in life maintenance of adrenalectomized animals. Progesterone, for instance, which has the $-COCH_3$ side chain is fairly effective in this respect, although it lacks other types of corticoid activity. The presence of a hydroxy group on C 17, as is the case in three of the six chief corticoids, gives rise to a marked diminution of potency in the life maintenance and Everse de Fremery tests, but enhances activity with respect to carbohydrate metabolism.

One non steroid which exhibits a small amount of corticoid potency is,

on concentration of circulating antibody has not been settled, reports in the literature are conflicting Since inflammation of either traumatic or allergic *origin is equally inhibited*, it has been deemed unlikely that cortisone acts by blocking the antigen antibody reaction Cortisone has been used locally for inhibiting inflammatory changes, as for example by *subconjunctival injection* in iritis A more familiar application is the systemic use of ACTH in the treatment of extensive thermal burns A related specific action is the inhibition of hyaluronidase

In spite of the fact that cortisone diminishes or abolishes the manifestations of hypersensitivity local passive transfer of hypersensitivity by serum can be accomplished from cortisone treated donors, and to cortisone treated recipients, as demonstrated by positive Prausnitz Kustner reactions, and with no diminution in intensity resulting from the use of cortisone The question has been raised as to whether, by paralyzing natural defense mechanisms, cortisone may not accelerate the spread of infections A significant increase occurs in the rate of growth of mumps virus in developing eggs injected with cortisone (25)

Cortisone has an effect antagonistic to that of DOCA on plasma potassium Cortisone produces an elevation of plasma K^+ If both drugs are given together, the DOCA effect, depression of plasma K^+, predominates These observations were made in rats Cortisone does not, however, antagonize the cardiovascular renal effects of DOCA

A similar antagonistic effect is shown in the elevation of the electroshock seizure threshold in rats, which is elevated by DOCA, and restored to normal by ACTH or extracts of adrenal cortex This and other evidence indicates that DOCA inhibits the ACTH production of the anterior pituitary

Definite and verifiable information is still scant concerning the mode of action of cortisone (and of ACTH which presumably acts chiefly by increasing the output of cortisone and related metabolic hormones) in relation to the therapy of the collagen diseases The group of collagen diseases includes rheumatic fever rheumatoid arthritis, scleroderma periarteritis nodosa, disseminated lupus erythematosus dermatomyositis, and psoriasis This group of diseases is characterized by alterations of collagen connec

of plasma

in the

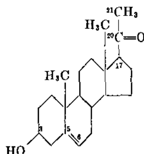
mally

tient s

effective In a manner not well understood cortisone enables ... tissues to repair the damaged mesenchymal system *Malkiel (36)*, however reports ACTH and cortisone to be without any apparent effect on the end results of histamine shock or of passive or active anaphylaxis in guinea pigs

when the hormone effects require moderation This would imply the ability for quick anabolism also, or the body could less well afford to be reckless with its supply of hormones

The manner and order in which the corticoids are formed in the adrenal cortex is still not known, although certain steps have been clarified by Pincus, Hechter, and their associates, utilizing the techniques of adrenal perfusion Their results have been summarized in a review (34) In brief, no steroid products are obtained from the perfusate of bovine adrenals unless either ACTH or a steroid is introduced Following the use of ACTH, a number of steroids could be identified in the perfusate, including cortisone In the absence of ACTH, but with single known steroids added to the perfusion fluid, introduction of hydroxyl groups, preferentially at C-11, but also at C 17 and C 21, could be demonstrated *Pregnenolone* (formula



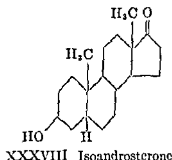
XXXIX Pregnenolone

XXXIX) added to the perfusion fluid yielded 17 hydroxycorticosterone, also corticosterone and progesterone This work as a whole leads to the strong supposition that ACTH promotes the formation of pregnenolone from a precursor, probably cholesterol

We have good evidence that the adrenocortical steroids are derived from cholesterol One of the points of evidence is that the adrenal cortex itself is particularly rich in cholesterol The adrenal cortex contains 3 to 4 grams of cholesterol per 100 grams of its wet weight, about 90 per cent of the cholesterol being esterified with fatty acids It also contains 300 to 400 milligrams of ascorbic acid per 100 grams wet weight The only other organ which exceeds these figures in content of cholesterol and ascorbic acid is the corpus luteum, which is also a producer of steroid hormones This high concentration of cholesterol and ascorbic acid in the adrenal cortex does not in itself constitute proof of the formation of the cortical steroid hormones from these substances and in fact there are several counts against ascorbic acid as a steroid precursor—patients with clinical scurvy show no characteristic signs of adrenocortical deficiency, ACTH delays

interestingly enough, a stilbestrol analog in which one phenol group has been replaced by the characteristic corticoid side chain, $-COCH_2OH$ Its activity is only about one two hundredth that of synthetic desoxycorticosterone acetate (35)

Metabolism. The 17 ketosteroids of the urine have their origin in corticoids as well as in androgens, and, in fact, arise predominantly from the former This last is demonstrated by the fact that in Addison's disease, which involves hypofunction of the adrenal cortex, 17 ketosteroid excretion drops well over 50 per cent (58) The 17 ketosteroids contain an additional oxygen group (either hydroxy or keto) on carbon 3, and sometimes on carbon 11 as well A typical urinary 17 ketosteroid is *isoandrosterone* (formula XXXVIII), which differs from androsterone only in the geometrical configuration of the hydrogen on carbon 5 The particular corticoid pre



XXXVIII Isoandrosterone

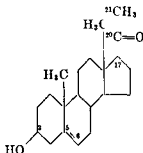
cursor of any particular 17 ketosteroid, or the manner in which the 2 carbon side chain on carbon 17 is degraded is not known

Cortical metabolites other than the 17 ketosteroids have also been found in the urine These are, generally, reduced corticoids differing mainly in the nature of the grouping on carbon 17 Compounds containing $-OH$, $-CHOHCH_3$ and $COCH_3$ have been isolated One of these is pregnanediol which is the chief urinary metabolic product of progesterone There is some evidence that corticoid pregnanediol may arise not only from the progesterone of cortical origin but from the degradation of desoxycorticosterone as well (17)

The steroid hormones in general are undoubtedly being actively metabolized at all times Whereas up to about 30 mgm of steroid hormones are excreted per day in males and non pregnant females and up to about 200 mgm in pregnant females, extremely minute quantities of active hormones amounting to one part in 40 000 to 15 000 000 parts of steroid secreting glands are present in the glands at any one time In view of the tremendous activity per unit weight of these hormones it must be useful for the body to be able to degrade the steroids and "quench" their activity rapidly

when the hormone effects require moderation This would imply the ability for quick anabolism also, or the body could less well afford to be reckless with its supply of hormones

The manner and order in which the corticoids are formed in the adrenal cortex is still not known, although certain steps have been clarified by Pincus, Hechter, and their associates, utilizing the techniques of adrenal perfusion Their results have been summarized in a review (34) In brief, no steroid products are obtained from the perfusate of bovine adrenals unless either ACTH or a steroid is introduced Following the use of ACTH, a number of steroids could be identified in the perfusate, including cortisone In the absence of ACTH, but with single known steroids added to the perfusion fluid, introduction of hydroxyl groups, preferentially at C-11, but also at C-17 and C 21, could be demonstrated *Pregnenolone* (formula



XXXIX Pregnenolone

XXXIX) added to the perfusion fluid yielded 17 hydroxycorticosterone, also corticosterone and progesterone This work as a whole leads to the strong supposition that ACTH promotes the formation of pregnenolone from a precursor, probably cholesterol

We have good evidence that the adrenocortical steroids are derived from cholesterol One of the points of evidence is that the adrenal cortex itself is particularly rich in cholesterol The adrenal cortex contains 3 to 4 grams of cholesterol per 100 grams of its wet weight, about 90 per cent of the cholesterol being esterified with fatty acids It also contains 300 to 400 milligrams of ascorbic acid per 100 grams wet weight The only other organ which exceeds these figures in content of cholesterol and ascorbic acid is the corpus luteum, which is also a producer of steroid hormones This high concentration of cholesterol and ascorbic acid in the adrenal cortex does not in itself constitute proof of the formation of the cortical steroid hormones from these substances, and in fact there are several counts against ascorbic acid as a steroid precursor—patients with clinical scurvy show no characteristic signs of adrenocortical deficiency, ACTH delays

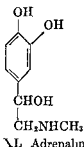
the progress of experimental scurvy in guinea pigs and administration of ACTH, but not of cortisone, is followed by an initial increase in urinary ascorbic acid On the other hand, positive evidence is afforded by the observations that whenever, by an effective means, the rate of formation of the adrenocortical steroids is increased in an experimental animal, a decrease can be measured in the cholesterol and ascorbic acid of the adrenal cortex

There are several ways in which the output of steroid hormones can be increased One very obvious method is by the administration of the pituitary adrenocorticotrophic hormone, ACTH If the experimental animal still has an intact pituitary, numerous other procedures will increase the output of corticoids The animal may be subjected to any type of stress such as heat, cold, trauma, or hemorrhage The result of any painful or traumatic stimulus if the pituitary is present and functional, is the same as if ACTH has been given the cholesterol and ascorbic acid of the adrenal cortex will decrease The same result can be accomplished by the injection of adrenalin, so it is quite possible that all painful and traumatic stimuli act by stimulating the secretion of adrenalin Not only is the pituitary necessary for this chain of events, but also probably the hypothalamus Therefore, the current concept is that the liberation of adrenalin stimulates the hypothalamus, which in turn stimulates the anterior pituitary, liberating the adrenocorticotrophic hormone All this can be done in the experimental animal

In man, direct analysis of the adrenals is impossible, but administration of ACTH decreases the level of cholesterol esters in the blood plasma In the human subject, subjected to traumatic or painful stimuli, a decrease in circulating eosinophils and an increase in the urinary excretion of 17 ketosteroids can be demonstrated It is of course true that the 17 keto steroids are not exclusively an end product of the metabolism of adrenal steroids They are also produced by the metabolism of testicular hormones It is possible, in a rather incomplete fashion however, to fractionate and differentiate those which are adrenal and those which are testicular in origin Other indices of adrenal activity can be used, such as decrease in circulating eosinophils Evidence of adrenal stimulation can be found in the human subject given adrenalin or insulin

It is not necessary to submit the human subject to actual trauma or actual pain to gain evidence of increased adrenal activity One can simply take the human subject and make him work hard make him do work which calls for careful and quick judgment Aircraft pilots show an increased 17 ketosteroid excretion during routine flights Test pilots during flights which are sometimes distinctly not routine show even greater increases in 17 ketosteroid excretion The ordinary taxpayer shows an increased 17

ketosteroid excretion during the time that he is awake and active, whatever kind of work he does compared with the rate of excretion while he is asleep Any activity which calls for attention and adjustment appears to work through this same mechanism stimulating the adrenal cortex to increased hormone output The schizophrenic contrariwise shows a con stant moderately low level of 17 ketosteroid excretion regardless of whether he sleeps or wakes regardless of his state of apparent agitation The neces sity for judgment discrimination and intellectual activity is not there Therefore the schizophrenic lacks the ups and downs of 17 ketosteroid excretion which the ordinary citizen has and which those engaged in stress ful occupations have to a still greater extent For the experimental work just described we are indebted chiefly to Pincus and Hoagland Their work (45) indicates that the difference between normal and schizophrenic



XL Adrenalin

adrenal responses is the result of failure in the response of the adrenal cortex to ACTH

OTHER HORMONES

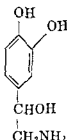
The adrenal medulla synthesizes a hormone which is neither a protein nor a steroid and which because of the very simplicity of its structure was the first hormone to be isolated and identified as a chemical entity back at the turn of the century The hormone is *adrenalin* or *epinephrine* (formula XL) and is a catechol derivative related to tyrosine and biosyn thesized from it (see Chapter 14)

Adrenalin secretion is stimulated by the sympathetic nervous system and the hormonal action on muscle and blood vessels is sympathomimetic Adrenalin causes liver and muscle glycogenolysis with resulting hyper glycemia and possibly glycosuria if there is glycogen in the liver (see page 487) It also increases oxygen consumption its effect in this respect being much more rapid than that of thyroxine Adrenalin is also considered to have a stimulating effect mediated by pituitary ACTH on adreno cortical activity Adrenalin contracts the arterioles increasing the blood

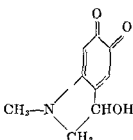
pressure The hormone is very active and the concentration in blood under normal conditions is one part in one or two billion In periods of stress, as in fear, pain, or anger, *there is hypersecretion of adrenalin into the* bloodstream with resultant blood pressure increase and glycogen break down enhancement in both liver and muscle so that the body is prepared better *to meet the emergency, either by flight or by physical resistance*

Adrenalin and the related compound *nor adrenalin* or *nor epinephrine* (formula XLI), represent the "sympathins" which are released by the *nerve endings of certain sympathetic nerves and which* may act as *chemical* transmitters of the nerve impulse In this respect, they and acetylcholine (see Chapter 3) may be termed in generalized fashion *neurohormones*

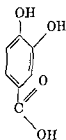
The effect of a single injection of adrenalin is of short duration *It is* quickly destroyed by oxidation in the capillary circulation through muscle



XLI Nor adrenalin



XLII Adrenochrome



XLIII Protocatechuic acid

and possibly other tissues but not in the liver Among the oxidation products have been identified *adrenochrome* (formula *XLII) and protocatechuic acid* (formula XLIII)

HORMONE-ENZYME RELATIONSHIPS

It has already been stated that the high activity of minute quantities of hormones made it seem likely that these chemicals exerted their influence through the activation or inhibition of enzyme *systems Unfortunately* evidence as to specific hormone-enzyme relationships is as yet scant Attempts at observing the effect of various hormones on specific enzyme systems *in vitro* have usually resulted *in flat failure or in the collection* of data of doubtful significance A reason for this may well be that the role played by hormones is not so simple as that of mediating a single reaction but is rather a complex one *involving control of the interrelationships* of many enzymatic reactions If that were the case a hormone would be expected to exhibit its effect only within the intact cell and this is indeed what seems to happen

Glucokinase

The nearest approach to an *in vitro* demonstration of a connection between a hormone and a specific enzyme system is in the case of glucokinase the enzyme which catalyzes the reaction of ATP and glucose to form ADP and glucose 6 phosphate (see Chapter 12) It was reported in 1945 (47) that this reaction could be inhibited by anterior pituitary extract and that such inhibition could be counteracted by insulin both *in vitro* and *in vivo* The particular anterior pituitary hormone involved in the inhibition is not yet certainly known but strong evidence points to the growth hormone (37) Later it was shown that adrenal cortical extracts could also inhibit the glucokinase reaction and that insulin could release this inhibition also As yet insulin has not been found to have an effect on any other enzyme involved in the glycose glycogen cycle and it should be stressed that even in the case of glucokinase the role of insulin is not one of stimulation but one simply of a release from inhibition If insulin has a more positive role it is either upon an enzyme system not yet studied or on intact cells only Stadie (54a) however has characterized as unsatisfactory all experimental evidence purporting to show physiologically significant effects of hormones on enzymes other than in intact cells He presents evidence to indicate that insulin acts by first combining with some component of the cellular structure and suggests that some anterior pituitary substance (not ACTH nor growth hormone) negates the effect of insulin by preferentially combining with the cellular component in question

Phosphorylase

Adrenalin in quantities too small to produce the characteristic rise in blood pressure will bring about breakdown of glycogen in liver and muscle with concomitant rises in blood glucose and blood lactic acid (Glucose is the end product of glycogen breakdown in liver and lactic acid the end product of glycogen breakdown in muscle) This action is very rapid the glucose and lactic acid of blood showing significant increases within three minutes Together with these phenomena there is a decrease of blood phosphate while hexose phosphate accumulates in muscle These various phenomena could all be explained if one were to suppose an increased activity of phosphorylase in the tissues as a result of adrenalin injections since accelerated phosphorolysis of glycogen would lower the concentration of glycogen and phosphoric acid and would raise the concentration of glucose hexose phosphate and lactic acid

Two forms of phosphorylase have been isolated from muscle phosphorylase *a* and phosphorylase *b* Phosphorylase *a* is obtained from muscle of deeply anesthetized animals and is active without addition of adenylic acid Phosphorylase *b* is obtained from fatigued muscle and is inactive unless

adenylic acid is added *In vivo* there is apparently an equilibrium between the two forms Evidence has been presented (54b) to show that adrenalin shifts this balance in favor of the active enzyme, phosphorylase *a* thus accelerating glycogen breakdown and initiating the other chemical changes mentioned above

In Vivo Experiments

In vivo studies have yielded some information on hormone-enzyme relationships (37) although the data obtained are less direct and specific than those of the *in vitro* demonstrations discussed On the other hand, the results are less liable to be considered artifacts not representing the true physiological state of affairs *In vivo* experiments may be carried out by analyzing various excised tissues of laboratory animals for certain enzymes, both under normal conditions and under conditions of unusual hormone stimulation (either endogenous as in pregnancy, or exogenous as after stilbestrol administration) or unusual hormone lack as after castration hypophysectomy, or adrenalectomy The variations in enzyme pattern from the normal in the case of the hormone-stimulated or unstimulated tissue may indirectly indicate the hormone's specific effect or effects on metabolism

Thus, after castration the alkaline phosphatase, acid phosphatase, succinic dehydrogenase, and cytochrome oxidase decrease in the rat, but not all to the same degree Injection of rat castrates with testosterone propionate maintains the normal enzyme pattern The liver tissue of diabetic rats has a higher concentration of succinic dehydrogenase and of the enzymes involved in glycolysis as compared with normal rats

Umbreit (55a), in studying the effect of adrenalectomy on the rat, reports that the ability of rat kidney to oxidize proline disappears three days after the operation but can be maintained or restored by cortisone Proline oxidation in the liver, however, continues entirely independently of cortisone Umbreit terms the situation "curious" since the enzymatic pathways of proline oxidation are, as far as is known, identical in the two tissues, although he warns that proline oxidase of neither tissue has yet been isolated and that the two may be found to differ subtly once characterized D Amino acid oxidase presents just the reverse picture It is under cortisone control in liver and not in kidney Umbreit believes that in neither case does cortisone act to activate an enzyme but rather that it acts to control the quantity of enzyme present

It can be seen from this section that the whole subject of hormone enzyme relationships is nearly a closed book The few glimmers of light that are now beginning to appear, while interesting and hopeful, are far from *sufficient to illuminate even a corner of the* subject

REFERENCES

1 BASSETT A M *et al* Protein bound iodine in blood V Naturally occurring iodine fractions and their chemical behavior Am J M Sc 202 516-527 1941

2 BLOCH K The biological conversion of cholesterol to pregnanediol J Biol Chem 157 661-666 1945

3 BRAND E Amino acid composition of simple proteins Ann New York Acad Sc 47 187-228 1946

4 BRAND E *et al* On the structure of thyroglobulin J Biol Chem 128 xi 1939

5 BRINK N G *et al* The activity of a hydrolysate of adrenocorticotropic hormone in rheumatoid arthritis J Am Chem Soc 72 1040-1041 1950

6 CALLOW N H *et al* Colorimetric determination of substances containing the grouping $-CH_2 CO-$ in urine extracts as an indication of androgen content Biochem J 32 1312 1331 1938

7 CANTAROW A *et al* Studies on inactivation of estradiol by the liver Endocrinology 33 309-316 1943

8 CANTAROW A AND TRUMPER M *Clinical Biochemistry* 4th ed Philadelphia Penn W B Saunders Co 1949

9 CHOW B F *et al* Effects of digestion by proteolytic enzymes on the gonadotropic and thyrotropic potency of anterior pituitary extract J Endocrinol 1 440-469 1939

10 CLAESSON L *et al* Crystalline human chorionic gonadotropin and its biological action Acta endocrinol 1 1 18 1948

11 COHN I J *et al* Studies in the physical chemistry of insulin II Crystallization of radioactive zinc insulin containing two or more zinc atoms J Am Chem Soc 63 17 22 1941

12 DEROBERTIS E Proteolytic enzyme activity of colloid extracted from single follicles of the rat thyroid Anat Rec 80 219-231 1941

13 DORFMAN R I Biochemistry of androgens *in* Pincus G and Thimann K V eds *The Hormones* Vol I New York N Y Academic Press pp 464 548 1948

13a EDINGER T The pituitary body in giant animals fossil and living A survey and a suggestion Quart Rev Biol 17 31-45 1942

14 ENGEL P AND ROSENBERG E Estrogen inactivating liver extracts Endocrinology 37 44-46 1945

15 FIESER L F Production of cancer by polynuclear hydrocarbons *in* University of Pennsylvania Bicentennial Conference *Cause and Growth of Cancer* pp 1 27 Philadelphia Penn University of Pennsylvania Press 1941

16 FRAENKEL CONRAT H *et al* The effect of cysteine on gonadotropic hormones J Biol Chem 130 243 249 1939

17 FRASER R W *et al* Colorimetric assay of 17 ketosteroids in urine J Clin Endocrinol 1 234-256 1941

18 FRED L *et al* Development of active pulmonary tuberculosis during ACTH and cortisone therapy J A M A 147 242 246 1951

19 GREER M A AND ASTWOOD E B The antithyroid effect of certain foods in man as determined with radioactive iodine Endocrinology 43 105-119 1948

20 GURIN S Carbohydrates of the gonadotropic hormones Proc Soc Exper Biol & Med 49 48-50 1942

21 GURIN S *et al* The gonadotropic hormone of urine of pregnancy II Chemical studies of preparations having high biological activity J Biol Chem 133

467–476, 1940 III Evidence of purity obtained by studies of electrophoresis and sedimentation *Ibid*, 477–484
22 HENCH, P S, *et al* Effect of cortisone and ACTH on rheumatic diseases J A M A, 144: 1327–1335 1950
23 HOLTROFF, A F, AND KOCH, F C The colorimetric estimation of 17 ketosteroids and their application to urine extracts J Biol Chem, 135: 377–392, 1940
24 KENDALL, E C The function of the adrenal cortex J A M A, 116 2394–2398, 1941
25 KILBOURNE, E D, AND HORSFALL, F L, JR Increased virus in eggs injected with cortisone Proc Soc Exper Biol & Med, 76: 116–118, 1951
25a LARDY, H A AND FELDOTT, G Metabolic effects of thyroxine *in vitro* Ann N Y Acad Sci 54. 636–648, 1951
26 LEATHEM, J H, AND CRAFTS, R C Progestational action of desoxycorticosterone acetate in spayed adrenalectomized cats Endocrinology, 27: 283–286, 1940
27 LEVIN, L The isolation of α-estradiol from the urine of stallions J Biol Chem, 158. 725–726, 1945
28 L'HEUREUX, M V, *et al* A new preparation of the parathyroid hormone J Biol Chem, 168: 167 176, 1947
29 LI, C H Sulfur amino acids in growth and adrenocorticotrophic hormones Federation Proc, 5: 144–145, 1946
30 LI, C H, AND EVANS, H M Chemistry of anterior pituitary hormones *in* PINCUS, G, AND THIMANN, K V, eds *The Hormones*, Vol I, pp 631–694 New York, N Y, Academic Press, 1948
31 LI, C H, *et al* Adrenocorticotropic hormone J Biol Chem, 149. 413–424, 1943
32 LI, C H, *et al* Isolation and properties of the anterior hypophyseal growth hormone J Biol Chem, 159: 353–366, 1945
33 LI, C H, *et al* Interstitial cell stimulating hormone II Method of preparation and some physico chemical studies Endocrinology, 27. 803–808, 1940
34 LIEBERMAN, S, AND DOBRINER, K Biochemistry of steroids Ann Rev Bio chem, 20: 227–264, 1951
35 LINNELL, W H, AND ROUSHDI, I M Synthetic compounds possessing biological activity similar to that of desoxycorticosterone Nature, 148. 595, 1941
36 MALKIEL, S The influence of ACTH and cortisone on histamine and anaphylactic shock in the guinea pig J Immunol, 66: 379–384, 1951
37 MEYER, R K, AND MCSHAN, W H Hormone-enzyme relationships Recent Progr Hormone Res, 5. 465–515, 1950
38 MILLER, G L, AND ANDERSSON, K J F The molecular weight of insulin J Biol Chem, 144: 459–464, 1942 An ultracentrifuge study of reduced insulin *Ibid*, 465–473
39 MUNSON P L, *et al* Isolation of dehydroisoandrosterone sulfate from normal male urine J Biol Chem, 152: 67–77 1944
40 PEARSON, O H, AND ELIEL, M D Use of ACTH and cortisone in lymphomas and leukemias J A M A, 144: 1349–1353, 1950
41 PEARLMAN, W H The chemistry and metabolism of the estrogens *in* PINCUS G, AND THIMANN, K V, eds *The Hormones*, Vol I, pp 351–400 New York N Y, Academic Press, 1948
42 PEDERSEN BJERGAARD G AND PEDERSEN BJERGAARD, K Sex hormone analyses III Estrogenic and gonadotropic substances in the urine from a woman with normal menstrual cycles and normal pregnancies Acta endocrinol, 1 263 281, 1948

43 PIERCE J G AND DU VIGNEAUD V Preliminary studies on the amino acid content of a high potency preparation of the oxytocic hormone of the posterior lobe of the pituitary gland J Biol Chem 182 359-366 1950
44 PINCUS G New color reaction for certain urinary 17 ketosteroids Endocrinology 32 176-184 1943
45 PINCUS G AND HOAGLAND H Adrenal cortical responses to stress in normal men and in those with personality disorders I Some stress responses in normal and psychotic subjects Am J Psychiat 106 641-650 1950 II Analysis of the pituitary adrenal mechanism in man Ibid 651-659
46 PITT RIVERS R Mode of action of antithyroid compounds Physiol Rev 30 194 205 1950
47 PRICE W H et al The effect of anterior pituitary extract and of insulin on the hexokinase reaction J Biol Chem 160 633 634 1945
48 REINEKE E P et al The effect of progressive iodination followed by incubation at high temperature on the thyroidal activity of iodinated proteins J Biol Chem 147 115-119 1943
49 ROSS W F AND WOOD T R The partial purification and some observations on the nature of the parathyroid hormone J Biol Chem 146 49-58 1942
50 SAHYUN M Crystalline insulin of low zinc content J Biol Chem 138 487-490 1941
51 SALTER W T The chemistry and physiology of the thyroid hormone in PINCUS G AND THIMANN K V eds The Hormones Vol II pp 181-300 New York N Y Academic Press 1950
52 SALTER W T et al Goitrogenic agents and thyroidal iodine Their pharmacodynamic interplay upon thyroid function J Pharmacol & Exper Therap 85 310-323 1945
53 SCHWARTZMAN G Enhancing effect of cortisone upon poliomyelitis infection (Strain MEFI) in hamsters and mice Proc Soc Exper Biol & Med 75 835-838 1950
54 SEGALOFF A The effect of partial hepatectomy on the inactivation of α-estradiol Endocrinology 38 212 213 1946
54a STADIE W C The combination of insulin with tissue Ann N Y Acad Sci 54 671-684 1951
54b SUTHERLAND E W The effect of the hyperglycemic factor and epinephrine on enzyme systems of liver and muscle Ann N Y Acad Sci 54 693 706 1951
55 TALROG A et al The monoiodotyrosine content of the thyroid gland J Biol Chem 184 83 97 1950
55a UMBREIT W W The influence of adrenalectomy and cortisone treatment on enzymatic reactions in rat tissues Ann N Y Acad Sci 54 569-574 1951
56 VAN DYKE H B et al The isolation of a protein from the pars neuralis of the ox pituitary with constant oxytocic pressor and diuresis inhibiting activities J Pharmacol & Exper Therap 74 190-209 1942
57 VENNING E H Adrenal function in pregnancy Endocrinology 39 203 220 1946
58 VENNING E H AND BROWN J S L Excretion of glycogenic corticoids and of 17 ketosteroids in various endocrine and other disorders J Clin Endocrinol 7 79-101 1947
58a VENNING E H et al Isolation of androsterone sulfate J Biol Chem 146 369-379 1942

59 WAUGH, D F Regeneration of insulin from insulin fibrils by the action of alkali J Am Chem Soc, 70: 1850–1857, 1948
60 WEST, E, AND FEVOLD, H L A comparison of interstitial cell stimulating, ovarian stimulating and inhibiting actions of pituitary glands of different species Proc Soc Exper Biol & Med, 44. 446–449, 1940
61 WHITE, A, *et al* Prolactin J Biol Chem, 143: 447–464, 1942
62 WITSCHI, E The quantitative determination of follicle stimulating and luteinizing hormones in mammalian pituitaries and a discussion of the gonadotropic quotient, F/L Endocrinology, 27. 437–446, 1940
63 ZONDEK, B, AND SCHULMAN, F The mechanism of action and metabolism of gonadotropic hormones in the organism Vitamins & Hormones, 3 297–336, 1945

PART III

Growth

CHAPTER 7

Nucleoproteins and Growth

Growth in its most general sense is not characteristic of life alone. Crystals grow in evaporating solutions and deltas grow out to sea. These latter types of growth are, however, of the nature of physical accretions, where substances of a given type, such as KCl or fine silt, are added unchanged to an accumulation thereof. Living growth is characterized by the fact that substances *differing* from those composing the living organism are first changed to a suitable form and then assimilated. Living growth is also characterized, in multicellular organisms at least, by an increase in complexity with growth. Whereas a small crystal of potassium chloride can only grow to a large crystal of potassium chloride without changes in structure other than those involved in dimensional increases, the fertilized ovum grows, not to a large fertilized ovum, but to a complex and differentiated organism.

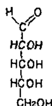
In considering the chemical basis of living growth it will be necessary to deal with *nucleoproteins*; these are compound proteins characterized by the possession of nucleic acids as prosthetic groups. *Nucleic acids* are complex substances among whose hydrolysis products are phosphoric acid (whence their acid reactions), pentoses, purines, and pyrimidines, the latter two being heterocyclic compounds the nature of which will be discussed. A detailed review of the chemistry of nucleic acids is that of Schlenk (34).

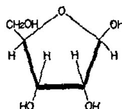
NUCLEIC ACIDS

Nomenclature

There are two general types of nucleic acids. One is found, for the most part, in the nuclei of cells, the other mainly in the cell cytoplasm. One of the chemical differences between these two nucleic acids is that the former type contains a desoxypentose, the latter a pentose. For this reason the two are termed *desoxypentosenucleic acid* and *pentosenucleic acid*. Where the desoxypentose and the pentose have been isolated and characterized, they have turned out to be *D-2-desoxyribose* and *D-ribose*, respectively (formula I). Since such isolation and characterization has been successfully

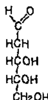
achieved for only a small number of nucleic acids, it may not be valid to conclude from it that ribose and desoxyribose are of universal occurrence in nucleic acids It is for this reason that the alternate names, commonly found in the literature, of *desoxyribosenucleic acid* and *ribosenucleic acid* are somewhat less precise than those which incorporate the word "pentose" Desoxypentosenucleic acid is most frequently abbreviated as DNA, less frequently as DRNA, or dorna Pentosenucleic acid is referred to as PNA or RNA

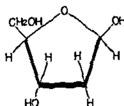




STRAIGHT CHAIN FORMULA RING FORMULA

D-RIBOSE





STRAIGHT CHAIN FORMULA RING FORMULA

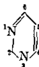
D - 2 DESOXYRIBOSE

I

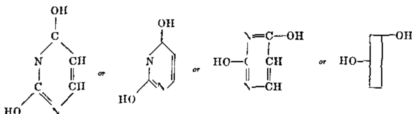
Earlier nomenclature of the nucleic acids was based on the sources from which they were derived and some acquaintance with the terms used for them is necessary if only because references to them in the literature might otherwise be confusing DNA is most easily isolated from cells high in nuclear content—glandular sources such as spleen or thymus being excellent for the purpose For this reason DNA has been frequently called *thymus nucleic acid* or *thymonucleic acid* Similarly, PNA is most easily isolated from cells poor in nuclear material and rich in cytoplasm, such as yeast, and has therefore been called *yeast nucleic acid* Again, because of their occurrence in the nucleus and cytoplasm, respectively, DNA and PNA are sometimes called *chromonucleic acid* and *plasmonucleic acid*, the

'chromo' prefix of the first term being a reference to the chromosomes of the nucleus, where the DNA is localized

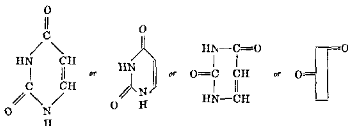
In this book, desoxypentosenucleic acid and pentosenucleic acid with the abbreviations DNA and PNA will be the terms used exclusively



II Pyrimidine



III Uracil (enol form)



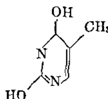
IV Uracil (keto form)

Hydrolysis Products

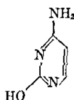
Pyrimidines. Among the hydrolysis products of nucleic acids are three pyrimidines A pyrimidine (formula II) is a heterocyclic compound characterized by the possession of a six membered unsaturated ring containing two nitrogens separated by a carbon The pyrimidines naturally occurring in nucleic acids are hydroxy and amino derivatives The hydroxy derivatives are usually presented in their tautomeric keto forms Thus *uracil*, 2 6 dihydroxypyrimidine could be pictured as the *enol* form (formula III), in which the double bond pattern of pyrimidine is maintained or the *keto* form (formula IV), involving a shift of two hydrogens and two double bonds A shorthand convention of drawing such formulas which makes

use of a simple rectangle to which the various substituents are attachec also used

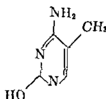
Two other pyrimidines occurring in nucleic acids are *thymine* (form V), which is 5 methyl uracil, and *cytosine* (formula VI), in which the 6 h droxy group of uracil is replaced by an amino group The distribution these pyrimidine bases (so called because of the weakly basic propert of the ring nitrogens) is important Cytosine occurs in both PNA and DN uracil in PNA only and thymine in DNA only No other pyrimidine occu



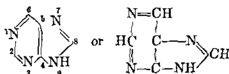
V Thymine



VI Cytosine



VII 5 Methylcytosine



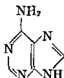
VIII Purine

in nucleic acids as a major component although small quantities of 5 *methyl cytosine* (formula VII) have been located in some nucleic acids (21) Thi amin (vitamin B_1), however, contains a 2 5-dimethyl 6 amino pyrimidin as part of its molecule, and somewhat more complex pyrimidine compound are the various synthetic barbiturates so important as sedatives, analgesics and anesthetics

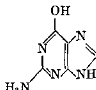
Purines Two purines are also found among the hydrolysis products of nucleic acids A purine (formula VIII) is a heterocyclic compound char acterized by the possession of two unsaturated rings one six membered and one five membered with two carbon atoms in common and two ni trogens in each ring

The two purines found in nucleic acids are *adenine* (formula IX), 6 amino purine, and *guanine* (formula X), 2 amino 6 hydroxypurine Both adenine and guanine are found in both PNA and DNA No other purines are found in any significant quantities in nucleic acids Three purines are, however, significant in the metabolism of nucleic acids These are *hypoxanthine* (formula XI), *xanthine* (formula XII), and *uric acid* (formula XIII), which are, respectively, 6 hydroxypurine, 2 6 dihydroxypurine, and 2 6 8 trihydroxypurine Uric acid is so called because of the weakly acidic properties of the hydroxyl groups, which are apparent if the formula is written in the enol form

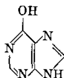
The alkaloids of tea cocoa and coffee are purine derivatives Among



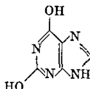
IX Adenine



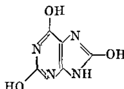
X Guanine



XI Hypoxanthine



XII Xanthine



XIII Uric acid

these are caffeine (1 3 7 trimethylxanthine) theobromine (3 7-dimethyl xanthine), and theophylline (1 3 dimethylxanthine) These are used as stimulants and diuretics

Nucleosides The combination of a nitrogenous base such as a purine or pyrimidine with a carbohydrate via an N glycoside linkage is termed a nucleoside In nucleic acids, purines and pyrimidines are so linked with carbohydrates, and by careful hydrolysis nucleosides can be separated The carbohydrates naturally occurring in nucleic acids are, as far as is known exclusively pentose or desoxypentose in nature, and where these have been characterized more closely they have proved to be D ribose and D 2 desoxyribose, the former occurring in PNA, the latter in DNA

Individual nucleosides derive their names from the purine and pyrimidine bases they contain, the purine nucleosides adding the suffix *osine*, the pyrimidine nucleosides the suffix *idine* The five nucleosides occurring in nucleic acids would thus be named *adenosine, guanosine, cytidine, uridine,*

and *thymidine* It should be especially noted that cytosine is the pyrimidine base itself, and not a nucleoside Nucleosides can, of course, be either *pentosides* or *desoxypentosides*, depending upon the nature of the carbohydrate moiety The time honored names such as adenosine, do not indicate in themselves the nature of the sugar, so that it is more accurate, although far less common, to speak of adenine pentoside and adenine desoxypentoside, rather than of adenosine The pentosides are more stable than are the desoxypentosides and where a commercial preparation of a nucleoside such as adenosine, guanosine or cytidine is obtained, it is almost invariably the pentoside

Two purine nucleosides, not occurring in nucleic acids, but nevertheless of interest in nucleic acid metabolism, are *inosine*, the pentoside of hypoxanthine and *xanthosine*, the pentoside of xanthine

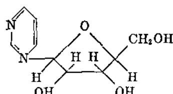
The glycoside linkage of a pyrimidine nucleoside (formula XIV) has been established at the nitrogen in the three position, while that in the case of the purine (formula XV) is at the nitrogen in the nine position. This last was determined spectrophotometrically (17) by comparing the absorption spectrum of a nucleoside with that of a 7 methyl purine (formula XVI) and a 9 methyl purine (formula XVII), since the nitrogens at positions seven and nine were the two possible choices for the link The spectrum of 9 methyl purine was similar to that of the natural nucleoside while that of 7 methyl purine was widely different

Nucleotides. The phosphoric ester of a nucleoside is a nucleotide The nucleosides of nucleic acids occur in combination with phosphoric acid and upon hydrolysis by alkali or by enzymes it is possible to obtain nucleotides as fragments The naturally occurring nucleotides obtained from the hydrolysis of nucleic acids are *adenylic acid*, *guanylic acid*, *cytidylic acid*, *uridylic acid*, and *thymidylic acid* The nature of the purine or pyrimidine base is in each case obvious from the name Again, the names do not in themselves indicate the nature of the carbohydrate portion so that it is more precise to speak of adenine pentose phosphate (or adenine pentose nucleotide) and adenine desoxypentose phosphate (or adenine desoxypentose nucleotide) As in the case of the nucleosides, the pentose nucleotides are much the more stable of the two types and are virtually the only ones met within the free state

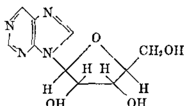
The point of attachment of the phosphate group to the nucleoside is always on the hydroxyl of one of the carbohydrate carbons, but is not invariably on a particular one Both adenosine-2 phosphate and adenosine 3 phosphate have been isolated from among the hydrolysis products of PNA, while adenosine-5 phosphate and its derivatives can be obtained from muscle This distinction between the sources of adenosine-3 phosphate and adenosine 5 phosphate has caused them to be referred to in

the literature, as *yeast adenylic acid* (formula XVIII) and *muscle adenylic acid* (formula XIX), respectively

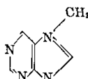
Nucleotides are not confined to nucleic acids ATP and ADP (see pages 144–147) are themselves nucleotides, while such coenzymes as DPN, TPN, FM, and FAD (see pages 215–217) are either nucleotides or dinucleotides



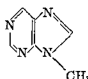
XIV Pyrimidine ribonucleoside



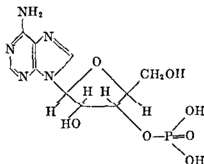
XV Purine ribonucleoside



XVI 7 Methyl purine



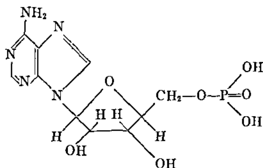
XVII 9 Methyl purine



XVIII Yeast adenylic acid

In all these cases the sugar is D ribose or, in the case of the flavins, the polyhydroxy alcohol derived therefrom, D ribitol In all but FM, adenine occurs, and in all the coenzymes another nitrogen containing ring system, either pyridine or isoalloxazine, occurs which is linked to ribose by the N glycoside linkage, which is characteristic of nucleosides and nucleotides

Oligonucleotides. Gentle hydrolysis of nucleic acids yields fragments still larger than the nucleotides just discussed These consist of a variable number of nucleotides connected by phosphate ester linkages These are referred to as *dinucleotides*, *trinucleotides*, *tetranucleotides*, and so on depending on the number of nucleotides present in the fragment As a group, these constitute the *oligonucleotides* Where the number of nucleotides contained in the fragment is large or indefinite, it is called a *polynucleotide* The nucleic acids themselves are in this sense polynucleotides



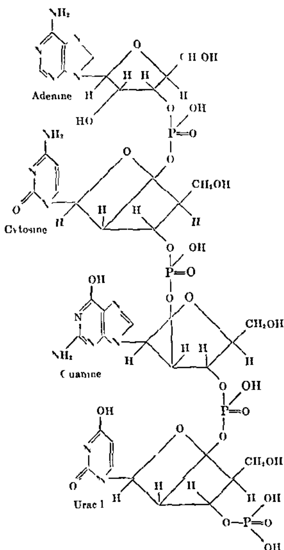
XIX Muscle adenylic acid

An important oligonucleotide, historically speaking is the *statistical tetranucleotide* a tetranucleotide containing one each of the four nucleotides found among the hydrolytic products of either PNA (formula XX) or DNA (formula XXI) (16) Each type of nucleic acid has its own statistical tetranucleotide, since the former contains only pentose nucleotides including uridylic acid while the latter contains only desoxypentose nucleotides, including thymidylic acid It was once thought that the statistical tetranucleotides represented the structures of the nucleic acids themselves *but this has been found to be a vast oversimplification*

Polynucleotide Structure

Nucleic acid molecular weights. Early studies on nucleic acids involved their isolation by alkaline extraction Under such conditions considerable depolymerization into relatively small oligonucleotides took place, so that the final product tended to have an average molecular weight roughly corresponding to that of a tetranucleotide, that is about 1200

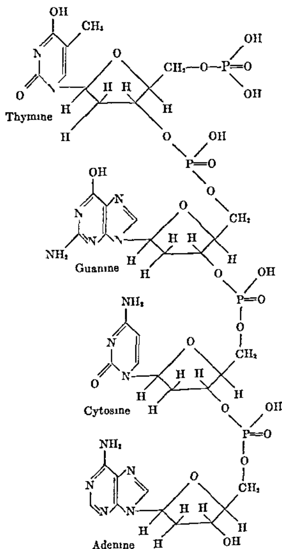
More recently, methods of extraction involving milder conditions, such as the use of neutral dilute saline solutions have succeeded in providing preparations more nearly native These were found to be astonishingly



XX PNA Statistical Tetranucleotide

high in molecular weight by experiments involving diffusion and the ultracentrifuge, while studies in viscosity and streaming birefringence showed them to be markedly asymmetric as well

Of the two, DNA is the more complex molecule, molecular weights up to 2,000,000 having been reported (35) For PNA preparations, values up to nearly 300,000 have been reported (12) In size, at least, nucleic acids are thus readily seen to be of the order of complexity of proteins



XXI DNA Statistical Tetranucleotide

From the pronounced asymmetry of the nucleic acid molecules, it is most often assumed that the molecules are an extended series of nucleotides linked through phosphoric ester bonds in the fashion indicated in the formulas of the statistical tetranucleotides A molecule of PNA with a molecular weight of 100 000 would have over eighty mononucleotides so connected, while a molecule of DNA with a molecular weight of 1,000 000 would have eight hundred mononucleotides so connected

Nucleic acid multiplicity So far we have presented a somewhat simple picture, analogous in a way to that of an extended polypeptide chain consisting of four different amino acids From what is becoming known of the diverse functions of the nucleic acids more complexity would be expected In two different directions, such expectations are being fulfilled

(1) The nature of the internucleotide linkage The general assumption that all the phosphate groups are present in their secondary state $\left(\begin{smallmatrix}\diagdown\\ \diagup\end{smallmatrix} PO_4H\right)$ is no longer as certain as it was once thought to be Titration data indicate that some samples of PNA possess primary phosphate groups at intervals of four nucleotides (18) Still other samples show a 25 per cent deficit in titratable hydrogen, indicating the presence of a branching nucleotide at intervals of four (34) (formulas XXIIa, XXIIb and XXIIc) Such one-every four variations in internucleotide linkage might explain the tendency for nucleic acids to break up into tetranucleotide fragments on alkaline hydrolysis X ray diffraction data indicate a periodic structure in the nucleic acid molecule with a pattern repetition every eight or sixteen nucleotides (2) Still another type of evidence in favor of something more than uniform and indefinite repetition of internucleotide linkages stems from the enzymatic depolymerization of nucleic acids Pancreatic nucleases will not hydrolyze the polynucleotide all the way to the individual mononucleotides but will leave behind undialyzable oligonucleotides of varying size, indicating different types of linkages (4)

(2) Base distribution Even when the statistical tetranucleotide had been abandoned as a possible representation of nucleic acids, it was tempting to keep it in an attenuated form by assuming nucleic acids to consist of repetitions of the tetranucleotide indefinitely extended The x ray diffraction data mentioned seemed to preclude random distribution of the purine and pyrimidine bases Furthermore, early analyses seemed to indicate equimolecular quantities of the four bases in each type of nucleic acid

More recently, paper chromatographic methods have been applied to the analysis of the purine and pyrimidine content of nucleic acids and have revealed that the distribution is not usually equimolecular (11) In a

XXIIa. Normal polynucleotides[1]
secondary phosphates, 100%

XXIIb Branched polynucleotide
primary phosphate, 25%
secondary phosphate 50%
tertiary phosphate, 25%

XXIIc Branched polynucleotide
secondary phosphate, 75%
tertiary phosphate, 25%

sample of yeast DNA for instance the molar proportions of adenine, guanine, cytosine and thymine were 1 8, 1 0, 1 0, and 1 9 respectively (44) In the PNA of dog pancreas, adenine, guanine, cytosine, and uracil were

[1] B represents purine or pyrimidine base, while S is the pentose or desoxy pentose sugar

found in molar proportions of 3 6, 8 8, 4 5, and 1 0, respectively In general, the distribution of bases tended to vary with the source of the nucleic acid Furthermore, enzymatic evidence would tend to indicate that base distribution is not only unequal but unperiodic as well Thus depolymerizing enzymes will split off fragments rich in pyrimidine nucleotides and leave behind relatively large portions that are high in guanine and adenine, particularly the former

From these considerations it is difficult to ignore the possibility that nucleic acids in general may equal proteins in complexity and specificity as well as in mere size Evidence from bacteriology indicates that varying bacterial strains do indeed possess specific nucleic acids, as distinct and characteristic of themselves as would be their proteins (3, 6) It is important to point out, in fact that at the moment less is known of nucleic acid "fine structure" than of protein "fine structure" and that even the primary internucleotide linkage, corresponding to the peptide linkage of proteins has not yet been finally elucidated

Occurrence

Chemical methods. Both DNA and PNA are of universal occurrence in all nucleated cells whether plant, animal or bacterial Even viruses, which are subcellular organisms, contain at least one or the other of the nucleic acids, and often both

Methods for detecting the gross quantity of nucleic acids in various tissues usually involve colorimetric determinations of either phosphate groups or of the sugar components of the nucleic acids In the former case, the tissue must be first fractionated in order to remove acid soluble phosphate and phospholipids which would otherwise interfere (36) In the latter case the two nucleic acids may be distinguished from one another by the use of appropriate reagents Desoxysugars are in general more active than their fully oxygenated relatives so that DNA will react with some substances with which PNA will not The most widely used chemical test for DNA consists of heating it in the presence of diphenylamine under appropriate conditions, yielding a blue color which is sufficiently delicate to detect as little as 20 mgm per ml (1) No color is obtained with PNA The specific detection of PNA is the harder problem Orcinol and phloroglucinol show reactions with both DNA and PNA, but are more sensitive with respect to the latter For a summary of chemical tests for nucleic acids, the student is referred to a review article by Schneider (37)

Nucleic acids have been found in greatest concentration in actively growing tissue and in glandular tissue such as pancreas and testis which have as part of their function the continual manufacture of protein in the form of enzymes or hormones

Ultraviolet microspectrophotometry. For the determination of the intracellular distribution of nucleic acids, ordinary chemical methods are insufficient Caspersson (10) has described a spectrophotometric technique which can record photographically the light absorption of various portions of tissue slices Ultraviolet light is used since the unsaturated ring systems of the purines and pyrimidines absorb strongly in the range between 250 and 280 millimicrons, and a microscope attachment allows individual cells to be photographed The only significant interference in this method is from the aromatic amino acids of proteins, since their ring systems also absorb in the indicated range At equivalent concentrations their absorption is only about 10 per cent that of purines and pyrimidines, so that in most cases protein interference can be ignored

Caspersson's results indicated the presence of nucleic acids both in the nucleus and in the cytoplasm, thus definitely establishing the fact that "nucleic acid" is somewhat of a misnomer and that they are not confined, as had been originally thought to the cell nucleus Furthermore, nucleic acids were not uniformly distributed throughout the cell In the nucleus, they are associated with the chromosomes, and in the cytoplasm they tend to exist as aggregates When such subcellular particles as mitochondria or microsomes are isolated by differential centrifugation of cytoplasm they are indeed found to be rich in nucleic acids

Histochemical stains. The intracellular location of DNA and PNA can be separately determined by the use of specific histochemical stains Of these the most famous is the *Feulgen stain* The reagent used is the red dye, basic fuchsin, which has been decolorized through addition of sulfurous acid If this is applied to tissue slices which have been exposed to the hydrolytic action of hydrochloric acid, the nuclear regions of each cell stain a brilliant red purple, since the desoxypentose freed by acid hydrolysis of DNA converts the decolorized basic fuchsin to a colored dye The less active pentose of PNA does not react in this manner

Both nucleic acids are basophilic, and dyes such as methylene blue will stain intracellular regions which are rich in either nucleic acid It is possible to locate PNA within the cell by staining serial slices before and after their exposure to some enzyme capable of catalyzing the depolymerization of PNA The enzyme ribonuclease is specific in this respect for PNA and will not affect DNA Once depolymerized, the PNA is easily washed out of the slice, and those regions which are stained before and not after enzymatic treatment are presumed to contain PNA (42)

On the basis of microspectrophotometry and histochemistry it has been fairly well established that DNA is located exclusively within the nucleus of the cell in close association with its chromatin, while PNA is found in the nucleolus and cytoplasm, and to a small extent in the chromosomes

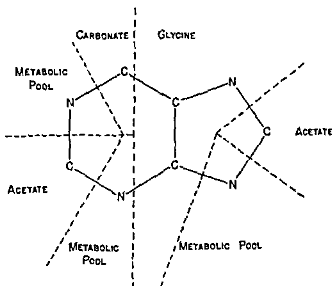
Nucleic Acid Metabolism

Although the word *metabolism* has been used frequently in previous chapters this is the first occasion where it has become necessary to deal systematically with a series of metabolic reactions It will be well, therefore, to define our terms Living growth, as has already been stated is a process characterized by the utilization of substances differing more or less from the substance in the growing body The body must be capable of taking the relatively complex components of its foodstuffs, converting them into smaller and simpler molecules, and then recondensing or refashioning these simpler molecules into complex substances that are characteristic of itself Chemical reactions in the body which form large molecules from small ones and which generally, store chemical energy in so doing are *anabolic* in nature The formation of glycogen from glucose and proteins from amino acids are examples of anabolism Such reactions are also referred to as *biosyntheses* In similar fashion, chemical reactions in the body which form small molecules from large ones and which, generally release chemical energy in so doing are *catabolic* in nature The utilization by the body of glycogen or fat to provide energy is an example of catabolism as are the various digestive processes It is obvious that in the normal mature body which is maintaining constant weight, the processes of anabolism and catabolism are on the average in balance During periods of growth, anabolic processes predominate, while under conditions of starvation the reverse is true The sum of all reactions which are either anabolic or catabolic in nature is indicated by the term *metabolism*

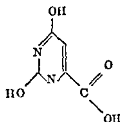
Anabolism In nucleic acid metabolism the reactions involving the purine fractions are best known The biosynthetic processes have been studied by feeding isotopically labeled substances to rats and then determining the isotope content of their tissue nucleic acids Strangely enough, labeled guanine when fed to these animals does not appear in nucleic acids and labeled adenine appears only to a small extent (one seventh of the amount fed) in the adenine of tissue nucleic acids and to a still smaller extent in the guanine (8) A large part of the labeled atoms, which are N^{15} in this case, appear in the form of allantoin, the final product of the degradation of purines in the rat Closely related purines such as xanthine, hypoxanthine, and isoguanine (2 hydroxy 6 aminopurine) after ingestion likewise appear mainly in excreted allantoin In general, it can be concluded that purines, as such after absorption into the body take part only in catabolic processes, while the purines of nucleic acids are almost exclusively the product of biosynthesis from simpler substances

To determine the nature of the simpler substances various possible precursors labeled with C^{13} or N^{15} were fed to pigeons and their excreted uric acid was then broken down so that each atom in the purine ring could

be separately studied for the extent of labeling (38) In this way it was found that the carbons in positions 2 and 8 of the purine ring were derived from acetate ion, and the carbon in position 6 from carbonate ion The C—C—N group of positions 4, 5, and 7 were derived from glycine while



XXIII Precursors of specific portions of the uric acid molecule



XXIV Orotic acid

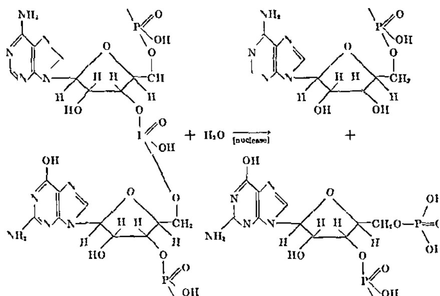
the other three nitrogens came from the general metabolic pool (see Appendix II) (formula XXIII)

Less is known concerning the biosynthesis of pyrimidines Purines are not pyrimidine precursors a fact known from the experiments already described involving ingestion of labeled purines Nor can ingested uracil cytosine, or thymine themselves be incorporated into nucleic acids although pyrimidine nucleosides and pyrimidine nucleotides can be so incorporated (33) Labeled orotic acid (uracil-4 carboxylic acid) (formula XXIV), after ingestion, has been found to increase the isotopic content

in the pyrimidines of liver PNA of rats, but not in the purines. This is not, however, considered a normal precursor. Studies on Neurospora indicate that there at least, pyrimidines arise from oxaloacetic acid, the ribose being attached before ring closure takes place (31).

The anabolism of the pentoses is dealt with in Chapter 12.

Catabolism. Ingested nucleic acids are broken down by a variety of digestive enzymes to smaller portions capable of being absorbed through the intestinal wall. The nucleic acids are freed from the nucleoproteins of



Portion of a polynucleotide chain

XXV

which they are the prosthetic groups by the action of the acid of the stomach and the proteases of the gastric and pancreatic juices. The polynucleotide structure of the nucleic acid is then hydrolyzed to smaller units by various nucleases (polynucleotidases). A nuclease is an enzyme which catalyzes the depolymerization of polynucleotides by splitting the C—O—P linkage between two adjacent nucleotides (formula XXV). For this reason they are spoken of as depolymerizing enzymes or *depolymerases*. From the nature of the reaction they catalyze, it can be seen that they are hydrolyzing enzymes and more specifically, phosphodiesterases. Nucleases are known which attack PNA only, and others which attack only DNA. The former is most frequently known as *ribonuclease*, the latter as *desoxyr*-

bonuclease or *dornase* The pancreas and pancreatic juice are particularly rich in both ribonuclease and desoxyribonuclease, and it is therefore probable that the first steps in nucleic acid depolymerization takes place in the duodenum Both nucleases have been prepared in crystalline form from beef pancreas (25, 26) Ribonuclease is the more stable of the two and has the rather low molecular weight, for a protein, of 15,000

Nucleic acids are not depolymerized all the way to the mononucleotide stage by pancreatic nuclease action Gutman (19) presents a scheme in which the nucleic acids are depolymerized only to various oligonucleotides by the depolymerases of pancreatic juice The oligonucleotides are then further hydrolyzed to the mononucleotide stage by the action of various specific nucleases in the intestinal mucosa Mononucleotides can be de phosphorylated by the alkaline phosphatase of the small intestine to the various nucleosides Both nucleotides and nucleosides can be absorbed in the intestine

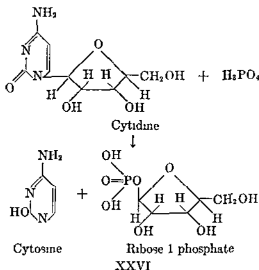
Of this absorbed material, pyrimidine nucleotides and nucleosides at least can be incorporated into nucleic acid to some extent The more general state of affairs is, however, a continued catabolism both for ingested nu cleotides and for degradation products of tissue nucleic acid This takes place chiefly in the liver

Nucleosides in general can be broken down to pentose (or desoxypentose) and purine (or pyrimidine) by the action of *nucleosidases* Traces of these are present in the small intestine which are specific only for purine nucleosides Purine nucleosidases are found in greater concentration in spleen, *lungs, liver, and heart Pyrimidine nucleosidases undoubtedly exist* but little is as yet known about them It has been shown that the action of *nucleosidase is not a hydrolysis but a phosphorolysis which results in* the production of base plus ribose 1 phosphate (23) (formula XXVI) For *this reason* Kalckar *suggests that the enzyme* be called *nucleoside phosphorylase* The reaction is reversible and its direction is controlled by the *concentration of free phosphate*

The further catabolic fate of two of the three fragments liberated by nucleosidase action, that is, pyrimidines and pentose (or desoxypentose), is wholly unknown The catabolism of *purines however, has been* elucidated Adenine and guanine are deaminated by the action of *adenase* and *guanase* to hypoxanthine and xanthine, respectively These purines can also be obtained by an alternative route in which adenosine and guanosine are first deaminated b [illegible] ctive *nucleoside deaminases* to inosine and xanthosine These latte [illegible] by purine nucleosidase to hypoxanthine and xanthine H [illegible] s oxidized in the liver to xanthine by th [illegible] *xanthine* [illegible] which also catalyzes the further oxid [illegible] e to uri [illegible] uric acid is the *final*

product of purine metabolism and is excreted in the urine as such (formula XXVII)

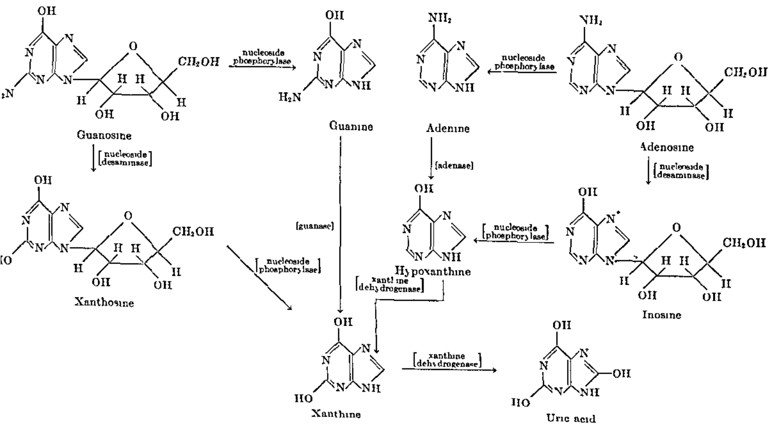
Gout. The occurrence of gout is the direct result of the fact that uric acid is the final product of purine metabolism in the human species Uric acid is highly insoluble in water, and to be removed successfully in the urine it must be present in low concentrations When, for any reason, uric acid (or perhaps closely related precursors) is produced in quantities greater than the kidney will excrete there may be deposition of the substance in the joint cartilages The joint usually first affected is that of the big toe This deposition has very painful consequences In one study six normal



Cytosine Ribose 1 phosphate
XXVI

subjects excreted a mean of 390 mgm of uric acid per day, while five young gouty patients excreted 567 mgm of uric acid per day (14) When both normal and gouty subjects were maintained on a purine free diet, the plasma uric acid concentrations were found to be 4 1 mgm per 100 ml for the normals and 7 7 mgm per 100 ml for the gouty cases Urinary uric acid outputs were 390 mgm and 567 mgm per 24 hours, respectively

The reasons for the increased activity of purine biosynthesis in the gouty subjects are not yet known, but gout would nevertheless be impossible with any naturally occurring rate of uric acid production if man were equipped with the enzyme *uricase* Uricase occurs in all species of mammals other than man and the anthropoid apes It is deficient, but not absent, in one breed of dog, the Dalmatian coachhound Uricase catalyzes the conversion of uric acid to allantoin (formula XXVIII) Allantoin, while

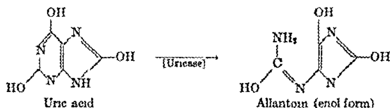


XXVII Scheme of purine metabolism in man

not soluble in the ordinary laboratory sense of the word, is despite that some twenty times as soluble as uric acid. At 20 C., 100 ml. of water will dissolve 60 mgm. of allantoin but only about 3 mgm. of uric acid.

It appears unfortunate that man lacks this useful enzyme, since its presence would involve no known disadvantages. Gout is thus a disease resulting solely from an evolutionary accident. Its incidence should be a forceful reminder that evolutionary forces, while given apparent direction by the competition to survive, depend upon the occurrence of random mutations which more often than not are undesirable.

Gutman (19) distinguishes between two varieties of gout: chronic tophaceous gout and acute gouty arthritis. That the manifestations of the former are due to the deposition of uric acid or urate in tissues he thinks certain, but he finds reason to doubt that urate is the actual villain in the latter case. Thus an acute attack of gout can not be induced in either normal or



Uric acid — Allantoin (enol form)

XXVIII

gouty subjects by feeding or injecting uric acid. Furthermore, in gouty patients acute gout often involves joints that do not show extensive urate deposits under x ray and, in fact, as urate accumulates the joint becomes less painful and acute symptoms are transferred to other joints as yet relatively free of urate deposits. Gutman concludes that acute gouty arthritis is evoked by some precursor of uric acid, as yet unidentified, rather than by uric acid itself.

The situation is made more serious and less amenable to correction by such a simple expedient as putting the patient on a low purine diet, since the body synthesizes uric acid from the simple molecules already discussed in connection with the biosynthesis of purines. Dietary lipid, protein, and carbohydrate can all be looked upon as uric acid precursors.

NUCLEOPROTEINS

Nature of Protein Moiety

In spermatozoa. Because the spermatozoön is little more than a small bag of tightly packed nucleoprotein (in the form of chromosomes) equipped with a cytoplasmic tail for locomotive purposes, it has been much used as

a source of DNA. For similar reasons, it has been possible to investigate the chemical nature of sperm desoxypentosenucleoprotein as a whole and that of fish sperm in particular because of the easy availability of large quantities thereof. It was early discovered that at least part of the protein of sperm desoxypentosenucleoprotein was highly basic and of exceedingly simple structure for a protein. Such proteins are, according to the classical classification, histones or even, in the case of the sperm of certain fish such as salmon or herring, protamines.

The protamines, in particular, are scarcely respectable proteins. The amino acid composition of salmine, the protamine of salmon sperm, has been elucidated (43); each molecule consists of but fifty-eight amino acid residues, no less than forty of which are arginine. The remaining residues consist of serine, proline, glycine, valine, isoleucine, and alanine. The molecular weight is about 8 000. Clupeine, the protamine of herring sperm, differs from salmine in that it contains threonine in place of glycine (5).

Other types of sperm cell studied contain histone. No known type of sperm contains both histone and protamine. The proportion of arginine in histone, while high enough to make the molecule markedly basic, is considerably lower than in protamines. Only 25 per cent of the histone nitrogen is attributable to arginine, as compared with 90 per cent in the case of protamines. Histones also contain tyrosine and sulfur containing amino acids, which protamines do not, and are large enough in molecular weight to be retained by a dialysis bag, which protamines are not.

The union between nucleic acid and the protamine or histone in sperm desoxypentosenucleoprotein is electrovalent in nature, the bond existing between the acidic negatively charged phosphoric acid groups of the nucleic acid and the basic positively charged guanidine groups of the protamine or histone arginine. As a result, the two substances can be separated relatively easily by varying the pH appropriately or by increasing the ionic strength of the solution.

Biochemists have been somewhat disturbed on occasion over the comparative simplicity of these protein constituents of spermatozoa. Stedman [illegible]

In genes. The nucleoproteins of somatic cells have been less well charac- terized than those of spermatozoa, largely because of the greater complexity of cellular composition and the lower concentration of nucleoprotein. The desoxypentosenucleoprotein of the cell nucleus has aroused particular attention because of its association with chromosomes, and the distinct possibility that the genes themselves are large but single nucleoprotein molecules.

The desoxypentosenucleoproteins of cell nuclei are usually isolated as nucleohistones and for long time it was thought that histones were the predominant proteins of somatic cell nuclei as they were of fish spermatozoa The possibility remained however, that nucleohistones were artifacts The large and relatively unstable native nucleoproteins might be disrupted into nucleic acid and protein moieties by the preparative techniques, however mild Where protein is not then removed by precipitating agents, nucleic acid when isolated would, due to its own acidic nature, tend to remove from the protein mixture the basic portions—that is the histones The association of the nucleic acid and histone would thus be purely a result of chemical manipulation and, in fact nucleohistones can be separated and recombined very easily by changes in acidity For this reason some have discarded the term nucleohistone as being too strongly indicative of a naturally occurring substance and have substituted the more artificial sounding term, *histone nucleate*

*In 1943, Stedman and Stedman (40) reported the isolation of the tryp*tophane-containing acidic protein mentioned above They gave it the name *chromosomin* It was found to be a constituent of all cell nuclei tested including those of sperm Their analyses show, for instance, that in the nuclei of ox spleen there is three times as much chromosomin as histone, while in cod sperm there is five times as much The Stedmans consider chromosomin to be the characteristic protein of the chromosomes and to be the substance which is the carrier of hereditary factors They have contended that nucleic acids and histones are too simple in structure to play a major role as hereditary factors and have even doubted the location of nucleic acid within the chromosomes despite the evidence of the Feulgen stain Their reasoning is that if the nucleic acids were soluble constituents of the nuclear sap they might combine with the Feulgen reagent to form a water soluble basic dye which would then combine with the acidic chromosomin of the chromosomes The final position of the color would thus indicate the site of protein occurrence only, not that of nucleic acids

This last contention has not met with general approval because of the growing conviction that nucleic acids are very far from simple in structure and because of the wealth of independent evidence in favor of the location of nucleic acid within the chromosomes Among such evidence is that derived from ultraviolet microspectrophotometry and also from the fact that upon microincineration of cells, phosphorus is detected in that portion of the ash corresponding to the chromosomes The existence of chromosomin itself, however has been confirmed by Mirsky and Pollister by isolation from nuclei of a similar protein which they called *chromosin* (29) It now seems most probable that the genes are *desoxypentosenucleochromosomin* in

nature, and that this substance, rather than the protein alone or nucleic acid with or without histone, is the carrier of heredity

In viruses. Further knowledge concerning nucleoproteins can be obtained from a study of viruses There is no glib general definition for the term *virus* It is applied to those agents of disease which (a) can pass through a standard filter capable of holding back bacteria, and which (b) fail to multiply in any medium other than living cells of a susceptible host With the advance of knowledge the boundary between viruses as so defined and bacteria proper has become less distinct Viruses thus vary in size and complexity of structure from the equivalent of large protein molecules almost to the equivalent of small bacteria For an introduction to viruses the student is referred to Burnet (9)

Chemically, all known viruses contain at least one type of nucleoprotein Many possess both types and various other constituents as well The advantage of viruses as a means of studying nucleoproteins resides in the fact that in viruses smaller amounts of non nucleoprotein material may be found as compared with amounts found in bacteria and other cells Indeed, the smaller viruses, particularly those infecting plants, are virtually pure nucleoprotein which can actually be prepared in crystalline form without destroying the infectivity of the molecule

The protein content (not counting the nucleic acid) of the viruses thus far studied is always more than 50 per cent In the case of the tobacco mosaic virus it is about 94 per cent (16) In all cases known the protein is not the basic histone type, but is a comparatively acidic, globulin like substance, much more closely resembling chromosomin The nucleic acid is bound to the protein, not by the electrovalent linkages characteristic of the isolated nucleohistones of sperm and cell nuclei but by primary covalent bonds often as firm as those which bind the heme of hemoglobin In many cases, separation of the nucleic acid from the virus protein can be accomplished only at the cost of denaturing the protein

In general, the exact relationship of nucleic acid to the protein of which it is the prosthetic group is by no means well understood Nucleic acids exhibiting extreme asymmetry can be derived from nucleoproteins of considerably less marked asymmetry The difference in properties is sometimes great enough to make it difficult to understand how a molecule of the nucleic acid can possibly fit within the nucleoprotein molecule from which it was isolated Thus it is pointed out that a molecule of histone nucleate from thymus with a molecular weight of about 2,000,000 can yield a molecule of nucleic acid with a molecular weight of 1,000,000 This nucleoprotein as a whole has dimensions calculated to be 154 8 millimicrons by 4 3 millimicrons, whereas the nucleic acid moiety once isolated turns out to have dimensions of 450 millimicrons by 1 6 millimicrons (16) Even

allowing for error in determinations of these values it remains a puzzle as to how to fit the long molecule into the short one The strong possibility that nucleic acids suffer changes in properties in the process of isolation, no matter how mild this may be is thus emphasized

Nucleoproteins and Autoreproduction

Living growth consists fundamentally of the synthesis of protein molecules Since protein molecules are so complex, the production of such molecules in the specific form required for a particular function in a particular tissue of a particular organ would seem a formidable undertaking The fact that specific protein molecules are produced innumerable times and almost always correctly is perhaps one of the most astonishing features of living tissue Furthermore, if we postulate that proteins are synthesized by the action of specific enzymes, we must ask what synthesizes these enzymes which are themselves proteins what synthesizes the enzyme synthesizers, and so on for as long as we have breath to ask To cut short this endless chain of questions we must postulate that some biochemical substance must have the property of *autoreproduction*—the ability to reproduce itself exactly when placed in the proper medium *The simplest substances known to be autoreproducible are the genes and the viruses and both of these are largely or entirely nucleoprotein in nature* To understand growth therefore, it will be helpful to consider the role of nucleoproteins as the entities which dictate in some manner the exact nature of the specific proteins to be produced

In nuclei A vast amount of indirect evidence (see Chapter 9) in the field of genetics has led to the conclusion that chromosomes consist of chains of smaller structures which act as carriers of hereditary traits These structures have been termed *genes* Although genes have never been isolated in the chemical sense, it is likely that they are complex desoxypentose nucleoprotein macromolecules The gene is characterized by two important properties

(1) It is autoreproducible In the process of mitotic division it duplicates itself *exactly* so that each daughter cell contains a set of identical genes (except in gametogenesis) In this way specific biochemical characteristics are perpetuated through the entire structure of a living organism

(2) It acts as a catalyst for the synthesis of a specific enzyme Experiments with the mold *Neurospora crassa* have shown that strains varying in their requirements for various food factors can be produced after exposure to ultraviolet light (7) The new requirement for a particular food factor arises from the failure of the altered strain of mold to perform a certain chemical synthesis that had previously given it no trouble This failure was undoubtedly due to the inability of the organism to synthesize

an appropriate enzyme A study of many such strains has resulted in the conclusion that the loss of the ability to synthesize an enzyme was due to the change induced in a single gene as a result of the absorption of an energetic ultraviolet photon Such experiments are considered to support the theory that each individual gene is responsible for the production of a single specific enzyme (7), and according to this "one gene one enzyme" hypothesis, the number of different enzymes in a cell and the number of functioning genes are identical

It should be obvious that if genes act as enzyme synthesizers, this in itself would enable them to control all species and individual differences Specific proteins are synthesized under the catalytic influence of specific enzymes The loss of a single enzyme, the one capable of oxidizing tyrosine to the catechol derivative, will result in an albino offspring

Any break in the hereditary pattern from parents to offspring which can not be accounted for by the simple reshuffling of genes inherited from mother and father (see Chapter 9) is termed a mutation The procedure mentioned, where under ultraviolet bombardment an individual neurospora capable of synthesizing for instance, the amino acid lysine from inorganic constituents produces asexually a new individual which is incapable of such a synthesis, is in effect an artificially produced mutation Most of the mutations dealt with in such experiments are fundamentally changes in specific gene molecules Such changes can be produced artificially by energetic radiation (ultraviolet, x rays, gamma rays neutrons, alpha particles) and also by a variety of chemicals such as colchicine, nitrogen mustards and methyl cholanthrene

The methods whereby mutations are produced in the laboratory are drastic ones which apparently render a gene unable to perform a function Spontaneous *back mutations* have been observed in Neurospora in which an altered gene regains its function or in which perhaps a second gene is so changed as to take over the role of the first Such spontaneous gains in ability are far less frequent than the induced losses The causes of "spontaneous" mutations in organisms during the course of evolution remain a field for speculation The effect of cosmic rays has been suggested as well as the radiation effects of radioactive isotopes of biochemically significant elements which either occur naturally, as K^{40}, or are continually produced in small quantities as a result of cosmic ray bombardment, as C^{14} Experimental proof for any of these hypotheses is as yet unsatisfactory

The question arises as to how, exactly, the gene desoxypentosenucleoprotein can direct either autoreproduction or enzyme synthesis Both are aspects of the same problem, since in each case the gene directs the synthesis of a specific protein or nucleoprotein Unfortunately, there is no satisfactory answer as yet As a result of x ray diffraction studies it has been found that

the desoxypentosenucleic acid is so arranged that the pyrimidine and purine bases thrust out from a rigid and inflexible backbone in such a fashion that if the backbone is considered as lying in the *plane* of the paper, the bases form parallel "plates," their rings lying in the plane *perpendicular* to that of the paper. The distance between each heterocyclic ring is calculated as 0.34 millimicrons, which is very close to the distance between the peptide residues in a polypeptide chain. The picture which can be drawn from this is that of a specific nucleic acid, the purines and pyrimidines of which act as a template along which amino acids may align themselves in an order dictated by the "fine structure" of the DNA to form a polypeptide chain. The protein thus formed would be one or more molecules of the enzyme for the biosynthesis of which the gene is the catalyst. The new molecule or molecules would then be detached and the process would begin again. An analogous procedure, in which nucleotides or their precursors are involved, might account for autoreproduction.

Ultraviolet microspectrophotometry has made it possible to study the DNA distribution within a chromosome. Caspersson's group has found that portions of the chromosome show a fine structure consisting of bands rich in nucleic acids, with the regions between relatively free of nucleic acid. The nucleic acid composes about 30 per cent of the banded regions, the absolute quantity within a single band being estimated at between five and fifty times 10^{-11} mgm. The remainder of the band is protein, rich in diamino acids, which may therefore be histone in character. The protein in the interband spaces is poorer in diamino acids and richer in tyrosine and tryptophane and thus more nearly corresponds to chromosomin. The banded regions of the chromosomes Caspersson calls *euchromatin*. Interspersed between euchromatin sections of the chromosomes are regions which lack the banded structure and which are termed *heterochromatin*. These are rich in proteins containing a high proportion of diamino acids.

During prophase the nucleus loses protein to the cytoplasm while the nucleic acid content increases slightly, soon reaching a maximum. In early prophase the nucleic acid protein ratio of the nucleus is approximately 1:20, in late prophase, 1:5, and at metaphase, 1:3. During telophase the reverse process takes place as the tight dense chromosomes of metaphase spread out and become distributed through a rounded nucleus once more, thus being accompanied by a proliferation of protein. The picture that can be drawn is that of the nucleic acids of chromosomes directing the synthesis of protein material up to maximum growth, then initiating the process of mitosis, during which the synthesized protein is squeezed out of the nucleus, the nucleic acids autoreproduce and two cells are formed, the nucleic acids of each beginning the process of protein synthesis anew.

The nucleolus, unlike the nucleus proper, contains PNA rather than

DNA Since PNA is the characteristic nucleic acid of the cytoplasm it has been suggested that PNA *is formed by the DNA of the genes* The question, however, of whether PNA is formed from DNA, or vice versa, or whether each is formed independently is still highly controversial It should be pointed out that PNA also occurs in chromosomes to the extent of about 10 per cent of the total nucleic acid (30) Since pentosenucleoproteins *can possess the property* of autoreproduction, as is demonstrated in tobacco mosaic virus, in the chromosomes they may synthesize cytoplasmic PNA

In cytoplasm. The PNA of cytoplasm, like the DNA of the nucleus, occurs largely in the form of particulate structures These structures include the *mitochondria*, which are comparatively large granules one to three microns in diameter, and the much smaller *microsomes*, which are 0 06 to 0 15 *microns in diameter and which may be particles of mitochondria* broken off in the process of isolation (15) The mitochondria, which have been much studied, are major components of the cell cytoplasm In the liver cell, mitochondria account for 30 per cent of the total cellular nitrogen They are large liponucleoproteins which contain the enzyme systems involved *in aerobic oxidations and oxidative phosphorylations The PNA* may act as a "skeleton" along which the enzyme molecules are arranged in some specific order and also as a catalyst for the synthesis of these enzymes and for autoreproduction as well

Evidence for the synthetic function of PNA resides in the fact that many investigators have found that tissue in a state of active growth *is particularly* rich in PNA Thus embryonic kidney tissue will absorb ultraviolet *much more strongly* than will adult kidney Further, a tissue of which only part is growing will show a localized concentration of PNA in the growing portion A plant rootlet, for instance, will absorb ultraviolet more strongly as the tip is approached and most strongly at the outer surface of the tip The same applies to cells which produce secretions rich in protein The cytoplasm *of nerve cells is also rich in* PNA and there is experimental

The relationship between the directive properties of the [illegible] chromosomes and those of the *PNA of the cytoplasmic granules is* not *certain The more accepted view has the genes as the only* primary autoreproducers in the cell Since the PNA of the cytoplasmic granules was formed under genic influence, its influence on protein synthesis is secondary Any change in the PNA structure of the cytoplasm would thus come under t[illegible] heading of a non hereditary change similar to cutting the tail off a rat [illegible]

cytoplasm from the nucleolus the daughter cells find the lost unit restored just as the tailless rat remains tailless but produces tailed offspring

Another point of view is that there are cytoplasmic hereditary factors more or less independent of the genes According to this view the cytoplasmic granules not only reduplicate themselves, but once altered they may continue to autoreproduce the new form not only in the cell itself but in the daughter cells after mitosis Such autoreproductive hereditary factors in the cytoplasm are called *plasmagenes*

In bacteria and yeasts While bacteria and yeasts do not have nuclei as highly organized as those found in the cells of animals and higher plants nuclear material does occur Bacteria can even be said to have genes 250 being reported present in the colon bacillus (6) The greater complexity of the nucleus in higher cells may simply be the expression of the need for a more refined mechanism for cell division as the number of genes to be transmitted in exact form becomes greater

Studies on bacteria have given rise to new conceptions as to the relative importance of the nucleic acid and protein moieties of the nucleoprotein molecule in the control of enzyme make-up The earlier view was that the protein was the complex and specific portion of the molecule while the contribution of the nucleic acid was comparatively minor being restricted perhaps to furnishing a framework for the genes The fact that spermatozoa, the prime carriers of at least those genic factors contributed by the male parent, seem poor in proteins (presumably in the interest of smaller mass and consequent greater mobility) by including a large proportion of histone while carrying nucleic acid in its full complexity, was somewhat disturbing to this line of thought So also were the developing ideas as to the multiplicity and specificity of nucleic acids Most startling however was the discovery that some biochemical characteristics of bacteria could be directed by an extract of DNA alone

It was long known that an extract prepared from a "smooth" variety of certain strains of Pneumococcus could induce the transformation of a rough variety of the same bacillus into a type-specific "smooth" strain, and that the new smooth strain bacillus would then breed true In 1944, it was found that the active principle of the transforming extract was a desoxypentosenucleic acid with a molecular weight of the order of half a million (3) It was easily inactivated by the action of desoxyribonuclease "Smooth pneumococci differ from the rough" strains by possessing a polysaccharide capsule they have a tendency to change to the "rough" form spontaneously Since only the DNA of smooth" pneumococcus can perform the transformation the experiment is evidence of the extreme specificity of nucleic acids

Boivin (6) has performed similar experiments on colon bacilli and con

cludes that DNA is the primary instrument of heredity, postulating at least one specific acid, for instance, for each strain of that bacillus

In virus. Viruses which have invaded a cell can impose their own synthesizing capacities upon it Thus, to cite an extreme example, a bacterium infected by a bacteriophage *consisting largely of desoxynucleoprotein will* cease manufacturing its own characteristic PNA and DNA, and will proceed to manufacture only viral DNA without any decrease in its metabolic *activity as measured by its rate of oxygen utilization*

Viruses, in their proliferation within susceptible cells, seem frequently to produce new strains in a process analogous to mutation, as indicated by changes in their infectivity Knight (24) undertook to analyze the amino acid contents of various strains of tobacco mosaic virus, and found that differences were profound Differences in amino acid content were not confined to one or two amino acids which were particularly reactive, but included almost all amino acids in one strain or another The changes in composition even amounted to complete loss of an amino acid or, even more surprisingly, the gain of a new amino acid such as histidine which parent strains had not possessed These marked changes under the mild conditions of spontaneous mutation lead to the thought that the primary change is a slight one in the virus nucleic acid, the catalytic properties of which would then be sufficiently changed to produce larger changes in the virus protein *synthesized*

Zahler (46) claims that in the relatively large bacteriophage viruses twenty five genes or their equivalents may be present He estimates 200 000 phosphorus atoms to be present in a T2 bacteriophage, and allows 8,000 per gene He considers the nucleic acid of a single gene to have a molecular weight of 3,000,000

In such simple plant viruses as tobacco mosaic virus, we have an example of a pure pentosenucleoprotein acting as a synthesizing catalyst Caspersson suggests that PNA is of particular value in synthesizing single protein molecules and is therefore sufficient for the small plant viruses DNA, on the other hand, is a synthesizer capable of producing a series of molecules *in fixed linear order, so that it is necessary in* the more complex viruses such as vaccinia and bacteriophage which seem to be organized systems of molecules containing proteins, both nucleic acids and various phospholipids Astbury's x ray diffraction data are not inconsistent *with this notion* since they show DNA to be, in addition to a much larger molecule, a far more rigid molecule than PNA, and hence more fixed in its configuration and presumably less liable to random changes between *autoreproductions* and better adapted to the more complicated protein syntheses

PROTEIN BIOSYNTHESIS

So far, nucleoproteins have been described as both autoreproducible and as agents for the synthesis of specific proteins. Two questions arise in this connection. First, how is the specificity of the nucleoprotein induced biosynthesis brought about? The only possible answer so far is that the gene (or virus) in its capacity as an enzyme displays the same order of specificity that many other enzymes do, and it is capable of catalyzing relatively few reactions and dealing with only certain specific substrates, to yield only one final product and no other. When the more general problem of exactly what in an enzyme governs its specificity is solved, the actions of genes may prove but relatively minor extensions of the answer.

The second question is a more concrete one. Disregarding all questions of specificity in proteins or the arrangements of amino acids, by what chemical reactions is a polypeptide chain built from amino acids? Even this considerably simpler problem has not yet been answered satisfactorily although much effort has been expended in its investigation.

Perhaps the simplest possible solution to the problem of protein synthesis involves the concept of the catalysis of reverse reactions by enzymes. It will be remembered (see Chapter 5) that an enzyme alters the rate on either side of the equilibrium point. Enzymes are known which hydrolyze proteins to amino acids. The equilibrium point in this general reaction



$$\text{Proteins} + H_2O \rightleftharpoons \text{Amino acid mixture}$$

is, of course, very far to the right under ordinary test tube conditions.

Early attempts at protein synthesis *in vitro* involved the treatment of concentrated solutions of the products of peptic or tryptic digestion of proteins with fresh pepsin or trypsin (45). A precipitate is formed which possesses polypeptide properties and in fact resembles denatured protein. This precipitate is called *plastein*. There was some hope, at first, that plastein was protein in the usual sense of the word. However, ultracentrifugal studies showed at least some specimens of plastein to be composed of molecules of less than 1,000 in molecular weight (13), which would be equivalent to a chain of less than 25 amino acid residues.

The utilization of such proteases as trypsin, pepsin, papain, and chymotrypsin in experiments on protein biosynthesis fails to duplicate the situation in the body. These are enzymes of relatively low specificity. The cell itself is equipped with a variety of dipeptidases which would be much more likely to be useful because of their greater specificity, in the synthesis of proteins. Suppose that a protein "template" exists in the cell (20)—that is, a protein or nucleoprotein which can serve as a mold for the assemblage of amino acids. A system would exist within the cell composed of the

original polypeptide template flanked by a string of free amino acids absorbed by the cell from the bloodstream This system would be most stable when most symmetrical, that is, when each amino acid residue of the template was flanked by an amino acid identical with itself The individual amino acids arranged in the proper order would then be condensed, one to each of its neighbors by the services of the appropriate dipeptidase, and two proteins would now exist where before there was only one

The main difficulty *involved in this concept is this Since a protein is* now generally considered to be a polypeptide chain coiled upon itself in a specific three dimensional fashion and held in place by various subsidiary *non peptide linkages, how can it uncoil itself to a simple chain or, at the* most, a surface one amino acid thick so that it may form a template and, having completed its task, coil back into the original molecule? Langmuir (27) believes that protein synthesis takes place in interfaces where some proteins are known to be able to form two dimensional films without loss of their specific properties

So far we have neglected the question of the energy involved in protein biosynthesis There is no question but that the hydrolysis of proteins releases energy and that the reversal of the process requires a corresponding input of energy (20) Energy metabolism in the living body usually involves high energy phosphate bonds, and it is only natural that the application of these very useful packets of energy be investigated in connection with protein synthesis Lipmann (28) has succeeded in synthesizing amides and substituted amides of carboxylic acids in the presence of pigeon liver extracts where ATP was part of the system, but not in its absence None of these involved the formation of true peptides, but the compounds formed, which included glutamine from glutamic acid and ammonia, and *hippuric acid from benzoic acid and glycine,* were close enough to make it possible to suppose that peptide formation could proceed analogously It is Lipmann's theory that the ATP, or equivalent high energy phosphate *donor, reacts first with the carboxyl group of the amino* acid to form the high energy acyl phosphate This in turn reacts with the amino group of another amino acid, forming a dipeptide and splitting out phosphoric acid *From the fact that Lipmann observed an equimolecular relation*ship between the amide formed and the free phosphate liberated he concluded that one high energy phosphate is utilized for each peptide bond formed

A flaw in the theory, which Lipmann admits, *is that whereas the* peptide bond contains 2 5 to 3 0 Kcal per mol, the high energy phosphate contains about 12 Kcal per mol The energy waste is sufficient to make one wonder whether it is a likely mechanism *for a biosynthesis* which must be proceeding continuously An alternative suggestion is that the energy

is derived from the phosphate of nucleic acids (30) This would involve the utilization of a low energy phosphate bond but this is not an impossible contingency since the energy content of a peptide bond is just about that of a low energy phosphate bond Furthermore such a concept would involve a much sought for concrete connection between nucleic acids and protein synthesis However there is as yet no experimental evidence in favor of this

For additional speculations on the subject of protein synthesis (and little more than speculations are yet possible) the student is referred to a review article by Northrop (32)

REFERENCES

1 Asimov I et al Ratio of desoxypentosenucleic acid to potassium in normal and malignant human tissue J Cell & Comp Physiol 37 355–364 1951
2 Astbury W T X ray studies of nucleic acids Symp Soc Exper Biol 1 66–76 1947
3 Avery O T et al Studies on the chemical nature of the substance inducing transformation of pneumococcal types Induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III J Exper Med 79 137 15 1944
4 Bacher J L and Allen, F W Solubility and sedimentation studies on certain pentose nucleic acids J Biol Chem 184 511–516 1950
5 Block R J et al Preparation and amino acid composition of salmin and clupein Proc Soc Exper Biol & Med 70 494–496 1949
6 Boivin A Directed mutation in colon bacilli by an inducing principle of desoxyribonucleic nature Its meaning for the general biochemistry of heredity Cold Spring Harbor Symp Quant Biol 12 7 17 1947
7 Bonner D Biochemical mutations in neurospora Cold Spring Harbor Symp Quant Biol 11 14 24 1946
8 Brown G B Studies of purine metabolism Cold Spring Harbor Symp Quant Biol 13 43–51 1948
9 Burnet F M Virus as Organism Cambridge Mass Harvard University Press 1945
10 Caspersson T O Cell Growth and Cell Function New York N Y W W Norton & Co Inc 1950
11 Chargaff, E et al The nucleotide composition of ribonucleic acids J Am Chem Soc 71 1513 1514 1949
12 Cohen S S and Stanley W M Molecular size and shape of the nucleic acid of tobacco mosaic virus J Biol Chem 144 589–598 1942
13 Ecker P G The ultracentrifuge study of plastein J Gen Physiol 30 399 1947
14 Friedman M and Byers S O Increased renal excretion of urate in young patients with gout Am J Med 9 31–34 1950
15 Green D E The cyclophorase system in Edsall J T ed Enzymes and Enzyme Systems pp 15–46 Cambridge Mass, Harvard University Press 1951
16 Greenstein J P Nucleoproteins Advances Protein Chem, 1 209–287 1944
17 Gulland J M and Holiday E R Constitution of the purine nucleosides IV

Adenosine and related nucleotides and coenzymes J Chem Soc, 765-769, 1936

18 GULLAND, J M, AND JORDAN, D O Structure of yeast ribonucleic acid Nature 161. 561-562, 1948

19 GUTMAN, A B Some recent advances in the study of uric acid metabolism and gout Bull New York Acad Med, 27: 144-164, 1951

20 HAUROWITZ, F *Chemistry and Biology of Proteins* New York, N Y Academic Press, 1950

21 HOTCHKISS, R D The quantitative separation of purines, pyrimidines and nucleosides by paper chromatography J Biol Chem, 175: 315-332, 1948

22 HYDÉN, H Protein and nucleotide metabolism in the nerve cell under different functional conditions Symp, Soc Exper Biol, 1: 152-162, 1947

23 KALCKAR, H M The biological synthesis of purine compounds Symp Soc Exper Biol, 1: 38-55 1947

24 KNIGHT, C A The nature of some of the chemical differences among strains of tobacco mosaic virus J Biol Chem, 171: 297-308, 1947

25 KUNITZ, M Crystalline ribonuclease J Gen Physiol, 24 15-32, 1940

26 KUNITZ, M Isolation of crystalline desoxyribonuclease from beef pancreas Science, 108: 19-20, 1948

27 LANGMUIR I Molecular layers Proc Roy Soc, London, A170. No 940 1 39 1939

28 LIPMANN, F Mechanism of peptide bond formation Federation Proc, 8 597-602 1949

29 MIRSKY, A E, AND POLLISTER, A W Chromosin, a desoxyribose nucleoprotein complex of the cell nucleus J Gen Physiol, 30. 117-147, 1946

30 MIRSKY, A E, AND RIS, H The chemical composition of isolated chromosomes *J Gen Physiol* 31. 7 18, 1948

[illegible] ...nthesis of [illegible] 1947 [illegible] K, ed [illegible] niversity Press, 1949

33 ROLL, P M *et al* The metabolism of yeast nucleic acid in the rat J Biol Chem 180. 333-340, 1949

34 SCHLENK, F Chemistry and enzymology of nucleic acids Advances Enzymol, 9. 455-535, 1949

35 SCHMIDT, G, *et al* Enzymatic dephosphorylation of desoxyribonucleic acids of various degrees of polymerization J Biol Chem, 127 251-259, 1939

36 SCHNEIDER, W C Phosphorus compounds in animal tissues I Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid J Biol Chem, 161: 293-303, 1945

37 SCHNEIDER, W C Nucleic acids in normal and neoplastic tissues Cold Spring Harbor Symp, Quant Biol, 12: 169-178, 1947

38 SONNE, J C, *et al* Biological precursors of uric acid I The role of lactate, acetate, and formate in the synthesis of the ureide groups of uric acid J Biol Chem, 173. 69-79, 1948 *II The role of lactate, glycine, and carbon dioxide as* precursors of the carbon chain and nitrogen atom 7 of uric acid *Ibid* 81-98

39 SPIEGELMAN, S, AND KAMEN, M D Genes and nucleoproteins in the synthesis of enzymes Science, 104 581-584, 1946

40 STEDMAN E AND STEDMAN E Chromosomin a protein constituent of chromosomes Nature 152 267 269 1943
41 STEDMAN E AND STEDMAN E The chemical nature and functions of the components of cell nuclei Cold Spring Harbor Symp Quant B ol 12 224-236 1947
42 STOWELL R E Nucleic acids in human tumors Cancer Res 6 426-435 1946
43 TRISTRAM G R Constitution of salmine Nature 160 637 194
44 VISCHER E et al Microbial nucleic acids The desoxypentose nucleic ac ds of avian tubercle bacilli and yeast J Biol Chem 177 429-438 1949
45 WASTENEYS H AND BORSOOK H The enzymatic synthesis of protein Phys ol Rev 10 110-145 1930
46 ZAHLER S A Nucleotide content of bacteriophage genetic units Science 111 210 1950

CHAPTER 8

Cancer

Cancer is commonly spoken of as a disease of growth This is true in rather an inverted sense The cancer cell is one which, although proficient at the art of dividing, has in some manner lost the mechanism of growth cessation It is around the ability to stop growing rather the ability to grow that the disease centers From the theoretical standpoint this should be emphasized

Clinically, the growth of malignant areas is their most outstanding characteristic and the most easily noticed Such growth is not in itself, however, truly characteristic Malignant growth does *not* proceed at a rate greater than that of normal growth Embryonic tissue grows as quickly Even in adult life, when visible growth seems to have ceased, many tissues retain the ability to regenerate rapidly Injury to the skin, for instance, will usually stimulate surrounding areas to rapid mitosis Such regenerative growth may take place at a rate equal to that of the most malignant tumors The big difference lies in this, that when the regenerating skin has accomplished its purpose, rapid growth ceases and mitosis proceeds only at the rate necessary to replace epidermis A skin cancer, however, continues growth indefinitely

Cancer is also a disease of organization In all multicellular organisms cell specialization exists The more complicated the organism, the greater the degree of specialization Various cells become more and more adapted to the proper carrying out of relatively few functions and sacrifice their independence in so doing The multicellular organism becomes a society of cells, few of which could exist independently for more than a few moments That this is the price paid for what we can not help but look at as "progress" in the evolutionary scheme of things is fairly obvious An analogy can be made between this state of affairs and various human societies We can compare a Stone Age tribal society and that of a modern city such as New York, and note that the greater comfort of the latter implies a greater interdependence of man upon man How many New Yorkers could long survive in a state of nature, no longer protected by the artificial safeguards built up by the co operation of a specialized society?

Unfortunately, the fact of organization among cells exposes new vulnerabilities. Cells are potentially immortal, as is shown in the case of unicellular organisms and in the germ plasm of multicellular organisms. Despite this potentiality among their individual members, cell societies in many cases age and die through breakdown of *their organization*. The analogy to a human society will hold here, too. A blizzard can paralyze city life for days, threaten mass starvation if needed food is delayed by impassable roads, and do infinite damage although its direct effect upon the health of the individual man and woman within the city may be nil.

Cancer generally involves a breakdown in the organization of the cell society. Cells which have become cancerous tend to lose by degrees their specialized function, and to multiply without regard to their own welfare or to that of the whole organism. A consequence, perhaps, of this reaction against specialization, this retreat to independence, is the property of transmissibility shown by many cancerous growths. That is, a tumor or a portion thereof can be removed surgically and implanted in another organism, and providing it can elicit from the new host a supporting structure of connective tissue and an adequate supply of blood vessels, it will continue to grow and multiply. Normal tissues when so transplanted grow little if at all, so that the phenomenon emphasizes the almost complete autonomy of cancer in relation to the organism. It is as though it were a portion of the flesh turned parasite.

To summarize all this, cancers may be defined as growths of cells which are largely or wholly independent of the organism which supplies their nutrition—cells possessing a frequently atypical structure and having no definite growth limits.

All tissues of all multicellular organisms are subject to cancer, although some are much more prone to the disease than others, and certain phenomena among unicellular organisms as well bear some resemblance to cancer.

Cancer is not the most general term applicable to these disorders of growth. *Tumor* (from the Latin *tumere*, to swell) may be applied to any swelling; *neoplasm* (from the Greek *neo* and *plasma*, meaning new formation) refers to non-physiological growth or multiplication of cells. *Benign tumors* are growths which, although they may continue to grow slowly over long periods of time, remain restricted in area, do not spread to other parts of the body, and have little or no tendency to recur after surgical removal. The dangers of benign tumors are limited to the effects of pressure and obstruction by the tumors. A *malignant tumor*, to which the term cancer should be restricted, is one whose growth is unrestricted and which has a tendency to infiltrate neighboring tissues, and as a result of transmission of cancer cells via the blood or lymphatic system even to invade far removed

portions of the body Portions of a tumor which have taken root in distant parts of the body are *metastases*, and a tumor resulting from such a process is a *metastatic tumor* The two main classes of malignant tumors are the carcinomas and the sarcomas *Carcinoma* is a tumor arising from epithelial tissue, whether pavement or glandular, *while sarcoma is one arising from* connective and muscle tissues Either name may be modified by a prefix designed to indicate the particular tissue from which the tumor derives

One highly important factor, perhaps obscured by the broad and general nature of these terms, must be emphasized before any further consideration of the subject takes place Cancer is not a single disease Cancers differ from one another in properties of all sorts, not only in accordance with the various tissues from which they originate but with the species or organism in which they occur To illustrate this, let us use a biochemical example In the rat, hepatoma cells are characterized by very low catalase activities as compared with cells of normal rat liver In the mouse, hepatoma cells also show decreases in catalase concentration but this is not as marked as in the rat Furthermore, the catalase deficiency in hepatomas varies with the particular strain of the rat

The importance of this generality in connection with *human* cancer is obvious In no other human disease has so much work been done on animals and so little on human beings It is easy, often too easy, to assume that results gained from a study of mouse cancer will be applicable to human cancer *An indication that this might not invariably* be so is the fact that although polycyclic hydrocarbons are the classical carcinogens for rats, mice, and rabbits, attempts to induce cancer by their use on monkeys have so far failed (28) With all this in mind, the student is cautioned that except where specifically stated otherwise the material in this chapter has been derived from animal experimentation

CHEMICAL CONSTITUTION OF CANCER CELLS

There is no one chemical constituent or group of *constituents* which may be found in all normal cells and which does not occur in all cancer cells, or *vice versa* Attempts to show that concentrations of certain cell constituents in cancer cells are generally higher or lower than in normal cells have been only partially successful, since to every general rule formulated, there *always turn out to be exceptions*

The morphology of the cancer cell, as well as its chemical make up varies with the stage and location of the disease There is a progressive change, involving a loss of organization of the cell *Cancer cells* are more [illegible] than are the normal tissue [illegible] malities in the struc[illegible] So marked are *the*

abnormalities that, despite attempts at chemical diagnostic methods the surest method of diagnosis of cancer remain the biopsy, the microscope, and the trained expert eye of the pathologist

To turn to more chemical considerations cancer cells usually have a higher water content the percentage of water generally increasing with the rate of growth This immediately is an example of a variation which need not be in any way significant with regard to cancer In general, normal cells which are actively metabolizing tend to have higher water contents than those whose role in the body is more sedentary and sluggish The water content of cancer cells may therefore be simply an attribute of *active* cells rather than of *cancer* cells A similar statement may be made about the fact that cancer cells tend to be comparatively rich in PNA a property they have in common with normally growing cells

There is probably no element organic grouping or molecule which could conceivably be found in tissues which has not been analyzed for over and over again in cancers Unfortunately the particular variations from the normal, found in such analyses, are not consistent nor are the reasons for the variations known An individual variation is sometimes correlated with a particular property of cancerous cells For example, a deficiency of calcium frequently is found in cancers of various types Experiments have shown that intercellular adhesiveness is dependent upon the presence of calcium (40), and calcium deficiency may therefore help to account for the lesser adhesiveness of cancer cells and the tendency of individual cells to break away from the cancerous mass and set up independently as metastases in other parts of the body The amino acid composition of the proteins of cancer cells shows no consistent difference from that of the proteins of the normal cells of their tissue of origin

Enzymology of Cancer

The enzyme pattern Greenstein (15) has suggested an approach which would take into consideration not single constituents but the over all make up of the cell with particular emphasis on those arbiters of chemical function the enzymes The specialization of various cells is reflected in the enzymes they contain so that to use Greenstein s examples, bone tissue and intestinal mucosa can be distinguished from other tissues by the fact that they are rich in alkaline phosphatase and poor in catalase and arginase The two can be distinguished from one another by the fact that intestinal mucosa is also rich in esterase while bone tissue is not Similarly, cardiac muscle and skeletal muscle can be distinguished by the markedly higher content of cytochrome oxidase in the former In general one can assume without much difficulty that each tissue has its own distinct enzyme pattern by which it can be differentiated from all other tissues The question then

arises as to whether there is any significance in the change of this pattern rather than in any of its components in cancer cells

The technical difficulties in the way of such investigations are manifold It is usually impossible to obtain a sample of a single tissue The analysis of epithelial tissue is easily rendered meaningless by the presence in the sample of an indeterminate amount of connective tissue which would itself posse s an entirely different enzyme pattern Muscle tissue analyses are thrown off by the presence of blood This list can be extended indefinitely The case with regard to cancers is considerably worse Cancers when of a size admitting of chemical analyses frequently contain sizable necrotic areas Because of the undiscriminating growth of a cancer without regard to vascularization inner portions may literally die of lack of food and oxygen even while the outer rim is still heedlessly expanding The inclusion of such metabolically inert material in the analysis of a cancerous mass would destroy the significance of the pattern Worse still since the growth of a cancer into adjacent normal tissue (a property known as invasiveness) is often ragged and irregular, it is frequently difficult to dissect out a cancer without including varying amounts of normal tissue which in the case of metastases may have an enzyme pattern radically different from the cancer s normal tissue of origin A great deal of work in the analysis of cancerous and normal tissue has been undertaken without sufficient consideration of such possible interfering factors and for this reason careful histological controls should be included in order that the analyses of any tissue contain as part of the data such information as the percentages of the whole which are cancerous normal tissue of origin connective tissue necrotic areas and so on This can be done only by the microscopic study of representative slices of the tissue to be analyzed

Certain general conclusions have been drawn from enzyme studies The loss of cellular specialization in cancer extends to specialized enzyme pattern Cancer cells arising from tissue which is unusually high in a given enzyme tend to show lower concentrations Similarly cancers become richer in enzymes in which the normal tissue of origin is unusually poor Furthermore this tendency would seem to be progressive and is more marked in the more advanced cancers and particularly so in cancers that have been transplanted from host to host a number of times (17) Thus whereas cancers in their early stages resemble their tissue of origin rather closely differences increase with the progression of the disease so that cancers of varying origin tend to converge and approach one another in enzyme pattern It is as though chemically as well as morphologically the cell retrogresses to a primitive and unspecialized ancestral pattern

Specific enzyme systems in cancer The most striking change among the individual enzyme systems of cancer cells is the decline in the conte it

of the various enzymes involved in aerobic oxidation, such as cytochrome oxidase, succinic acid dehydrogenase and D amino acid oxidase This is not to say that cancerous tissue is lower in these enzymes than is normal tissue in general We can only say that the content is substantially lower than that of the normal tissue of origin in virtually every case The heme containing enzymes are an extreme case of this The catalase activity of liver not only declines markedly in hepatomas but also decreases frequently under the influence of distant cancer in the organism which has not established itself in the liver (16)

Alterations in other enzyme systems are neither as uniform nor as marked in cancers taken in general The activity of enzymes which are involved in the metabolism of nucleic acids or of proteins continues high in most cancers Examples of such enzymes are the nucleases, arginase, certain peptidases, and xanthine dehydrogenase Hyaluronidase is not present in cancers in any unusual concentration This is significant since it has been suggested several times that hyaluronidase or some closely related enzyme might be partly responsible for the invasiveness of cancers (8)

Enzyme pattern of the host. A cancer represents, in a sense, an alien element within the body Although derived from the host's own cells, its autonomy of growth and its failure to fit itself to the needs of the body lead to conditions of sufficient abnormality to elicit specific responses from the host even at early stages of the disease Such systemic responses become significant where the tumor approaches 5 per cent of the body weight, but the nature of the responses is not sufficiently invariant to be of diagnostic value Reference has already been made to the fact that there is a decline in liver catalase under the influence of distant cancers In the mouse a wide variety of non liver cancers have been shown to accomplish this, and the effect was found to become more marked as the disease progressed Furthermore, the effect was reversible since removal of the cancer by surgery restored the catalase function of liver to normal

The interest in such responses to the presence of cancer by the host is great, since by observation of such responses one could conceivably detect a cancer before it had grown big enough for direct observation and all too frequently too big or too metastatic for treatment Unfortunately, periodic observations of liver biopsies for their catalase content is impractical as a routine clinical test for the presence of cancer

The tissue most easily observed is, of course, blood, and it is for this reason that host responses to cancer have been most sought for there Blood catalase activity frequently declines in the presence of cancer, but the change is so small as to be of little or no diagnostic value Anemia, which often accompanies cancer, has too many other possible causes to be diagnostic Differences in the properties of blood of cancerous and normal

patients are frequently reported in the literature As examples, the plasma of cancerous patients was reported to have a lower ability to decolorize methylene blue than did that of normal patients (4), serum albumin of cancerous patients usually coagulated more readily than did that of normal patients (22), and plasma of cancerous patients inhibited trypsin activity more than did that of normal patients (6)

All blood tests for cancer so far reported are far removed from perfection Comparatively large percentages of both false positives and false negatives occur and repeated tests of their efficiency by workers in the field have proven increasingly disappointing One study of five such tests (10) ended in the conclusion that none of them were suitable even as screening tests

Electrophoretic analysis of plasma for the purpose of cancer detection has been disappointing Except in multiple myeloma no distinctive cancer *patterns of plasma proteins have been found* In multiple myeloma the globulin concentration is typically increased This disease also offers an example where a urinary constituent reveals the presence of a particular cancer In that disease the urine may contain a group of proteins of an average molecular weight of 37,000, with the unusual property of precipitating from solution at 45 to 58°C, and of redissolving at 100°C These are called *Bence-Jones proteins*

A useful diagnostic tool for the detection of a single type of cancer involved the measurement of the acid phosphatase activity of blood serum (19) In cases of metastatic carcinoma of the prostate this is markedly increased

Metabolism of Cancer Cells

It is tempting to assume that there is something superior about the metabolism of cancer cells some greater versatility or new source of energy that gives them an unfair advantage over normal cells This is based on the undoubted facts that (a) cancer cells can invade and overgrow normal tissue, (b) cancer cells can grow at alien sites in the organism or even in alien organisms, and (c) cancer cells can multiply under conditions of food and oxygen supply that would be most unfavorable for normal cell growth These facts are not, however inconsistent with the thought that cancer cells prosper not through greater efficiency but through a capacity to adapt themselves to a 'lower standard of living' From this point of view, the cancer cell, through its loss of the high degree of specialization that characterizes normal cells no longer requires so specialized an environment and can therefore, despite a lowering of general efficiency compete favorably with its normal brother

The cytochrome system The oxygen consumption of normal tissue slices is markedly increased when excess metabolite, such as succinic acid

is added to the system Apparently, reserve supplies of cytochrome c and cytochrome oxidase are available to handle oxidation of the additional material Cancerous tissue slices show considerably less increase of oxygen consumption upon addition of metabolite It is almost as though, with characteristic improvidence cancer cells had no reserve supply of the cytochrome system and were content with whatever amount they could get by on A less anthropomorphic view is that cancer involves an impairment of the metabolic pathways involved in the biosynthesis of heme This is *rendered more plausible by the decrease of catalase activity and even of* hemoglobin in many cancers However, no conclusive evidence for this exists

In order to differentiate among various components of the cytochrome system, the oxygen consumption of tissue slices was studied after cytochrome c as well as metabolite had been added to the system (18) It was here found that the respiration of cancerous tissue approached that of normal tissue From this it is concluded that malignant tissue is relatively more deficient in cytochrome c than in cytochrome oxidase and that it is the concentration of the former that is the limiting factor under the conditions of the experiment

Glycolysis The deficiency of the cytochrome system, which limits the power of the cancer cell to conduct aerobic oxidations, is accompanied by a greater tendency for the cell to reply upon glycolytic mechanisms for necessary energy Glycolytic reactions are a comparatively primitive method of energy production and much less efficient than aerobic oxidation (see Chapter 12) All tissues can glycolyze cancers differ from most, but not all normal tissues in that they will glycolyze even in the presence of adequate supplies of oxygen While most of the experiments upon which these conclusions are based were conducted *in vitro*, utilizing such techniques as Warburg manometry (see Chapter 5), confirmatory *in vivo* evidence exists The pH of growing tumors *in situ* was measured (23), and was found to be distinctly lower than that of normal tissue, about 7 0 as compared with 7 4 The pH of the tumor was found to drop still lower, to as little as 6 3, after administration of glucose either subcutaneously or intraperitoneally, without any signs of systemic acidosis, thus indicates the drop in pH to be confined to the tumor This is strong evidence in favor of the *in vivo* glycolysis of tumors since glycolysis involves the production of lactic acid as an end product (through the hydrogenation of pyruvic acid in the reducing medium of tissue) which is a relatively strong organic acid and easily able to lower tissue pH by the amounts experimentally measured

Although this tendency in favor of glycolysis is so far the characteristic that most clearly differentiates the majority of cancers from the majority

of normal tissues it is still not the final specific difference so long sought by cancer investigators The degree of predominance of glycolysis does not consistently differentiate cancerous from normal tissue

CARCINOGENESIS

The study of cancer was profoundly stimulated when, in 1915 two Japanese workers first announced the artificial induction of cancer in an experimental animal This was done by applying coal tar to rabbits' ears over long intervals until skin cancers appeared at the sites of application This meant that investigators no longer had to confine their animal studies to the relatively few spontaneous cancers they might find but could produce the cancers in quantity and in accordance with need Furthermore they could study cancer development in all its stages from the pre-cancerous through the primary tumor to secondary transplants Again from a study of the chemical nature of the cancer-producing agents or carcinogens there was always the possibility that the metabolic changes involved in carcinogenesis might be understood

Unfortunately complications developed Various carcinogens show species and tissue differences in their actions Therefore, the application of the results of animal experiments to human cancer is unreliable A given carcinogen differs in its effect upon a given animal depending on the method of application the solvent used the interval between applications the age diet and physical condition of the animal, and so on It remains difficult therefore to interpret the results obtained in any general manner Lastly so many carcinogens have been described (approximately one thousand) of such varying chemical nature that generalizations as to their action is all but impossible

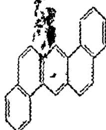
Non-Physiological Carcinogens

The term carcinogen may in its broadest sense be applied to any factor chemical or otherwise capable of inducing cancer Most of the agents used in the laboratory for the artificial induction of cancer are non physiological in the sense that the animal would not ordinarily be exposed to them in the natural course of its life Such agents fall into two chief classes—chemicals and short wave radiation

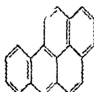
Polycyclic hydrocarbons The relationship between coal tar and carcinogenesis was first noted in the fact that skin cancer became an occupational disease among workers in the growing coal tar industry of the 19th century and as has been stated the first artificial cancers were induced by coal tar The isolation from coal tar of pure chemical carcinogens revealed the active substances to be polycyclic aromatic hydrocarbons To this

group belong a wide variety of active carcinogens which remain even today the most used agents for experimental carcinogenesis.

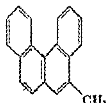
Two such hydrocarbons isolated from coal tar and found to be strongly



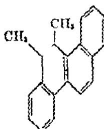
I 1,2,5,6-Dibenzanthracene



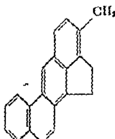
II 3,4-Benzpyrene



III 2-Methyl-3,4-Benzphenanthrene



IV 1,2-Dimethylchrysene



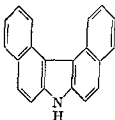
V 20-Methylcholanthrene

carcinogenic are *1,2,5,6-dibenzanthracene* (formula I) and *3,4-benzpyrene* (formula II). Other polycyclic ring structures, such as 3,4-benzphenanthrene and chrysene, which themselves were weakly carcinogenic if at all, yield active carcinogens upon alkylation. Thus *2-methyl-3,4-benzphenanthrene* (formula III) and *1,2-dimethylchrysene* (formula IV) are active carcinogens.

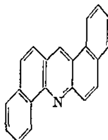
One of the most active, and certainly the most interesting, of this type

of carcinogen is *20 methylcholanthrene* (formula V), often called merely methylcholanthrene It should be seen at once that this hydrocarbon can be viewed as a steroid derivative in which rings A, B, and C have been aromaticized, and in which the C17 side chain has been condensed with ring C to form still a fourth benzene ring The name of the compound is derived from the steroid ring numbering convention (see page 117) which places the methyl substituent found on carbon 20 Methylcholanthrene can be prepared in the laboratory from desoxycholic acid, one of the bile acids

Heterocyclic analogs of the polycyclic hydrocarbons may also be carcinogenic The nitrogenous compounds 3,4,5,6-dibenzcarbazole (formula VI) and 1,2,5,6 dibenzacridine (formula VII) are examples These carcinogens are water insoluble and are either applied to the skin as solutions in benzene or other fat solvents, inducing carcinomas, or are injected sub



VI 3,4,5,6 Dibenzcarbazole



VII 1,2,5,6 Dibenzacridine

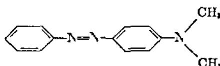
cutaneously as solutions in more physiological solvents such as lard or sesame oil, inducing sarcomas It must not be thought that cancer follows the touch of these compounds unfailingly or quickly Applications must be continued, usually over a period of months, for the effect to take place

The carcinogenic activity of a given compound varies with the size and frequency of the dose, the manner of application (whether painted or injected), the solvent used, the presence of additional substances not in themselves carcinogenic, and the nature of the animal's diet Thus 10-methyl 1,2 benzanthracene is highly active when administered subcutaneously, but only weakly active when applied to the skin while the reverse is true of 2 methyl 3,4 benzanthracene Although *1,2 benzanthracene* is not carcinogenic when applied externally or injected subcutaneously, it will cause hepatomas in some rats if included in their diet Again, methylcholanthrene in acetone is a more effective carcinogen than when dissolved in benzene, while in solution in anhydrous lanolin carcinogenic power is almost entirely lost The application of croton oil to areas of skin being painted with 3,4 benzpyrene hastens the development of cancer, while the

simultaneous use of brombenzene or unsaturated dibasic acids with that carcinogen retards the effect A high fat diet often facilitates artificial induction of cancers, while a diet low in the sulfur containing amino acids may retard the development of some kinds of induced cancers

Studies of carcinogens have revealed a form of variation of response more intriguing and perhaps more important theoretically than any of those listed so far When carefully inbred strains of mice were used as subjects for applications of carcinogens it was found that different strains varied in the frequency and rapidity with which individual members developed cancers of certain types These strains usually showed a higher incidence of spontaneous cancers of those types as well The use of animal strains of known cancer forming characteristics is thus highly important if significant results are expected in laboratory investigations

Micro-organisms exposed to dilute solutions of a carcinogen such as methylcholanthrene exhibit an accelerated mitotic cycle (34) On the other hand yeasts exposed to camphor, which is not ordinarily considered a



VIII p Dimethylaminoazobenzene

carcinogen show inhibited mitoses but continue growth to produce larger than normal cells which display morphological changes characteristic of cancer cells (37) Whether such cancer like phenomena among micro-organisms are truly analogous to cancer in the multicellular organism remains of course a matter for speculation such experiments seem to show cancer as a truly basic cell disease

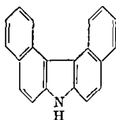
Other chemicals A chemical carcinogen second only to methylcholanthrene in experimental usefulness, is *p-dimethylaminoazobenzene* (formula VIII) a representative of a group of carcinogens completely different from the polycyclic hydrocarbons p Dimethylaminoazobenzene induced hepatomas in rats (but not in mice) when included in the diet The nature of the remainder of the diet affected tumor incidence carrots and rice stimulating the carcinogenic effect and liver depressing it This carcinogen is also frequently known as *butter yellow*

A related carcinogen of great versatility is *o-aminoazotoluene*, which not only induces hepatomas on feeding but also results in various forms of cancer when applied externally or injected subcutaneously It is effective on mice as well as on rats

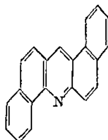
A peculiar carcinogen chemically in a class by itself is N *acetyl 2 aminofluorene* (formula IX) It is active only when administered orally,

of carcinogen is *20 methylcholanthrene* (formula V), often called merely *methylcholanthrene*. *It should be seen at once that this hydrocarbon can* be viewed as a steroid derivative in which rings A, B and C have been aromaticized, and in which the C17 side chain has been condensed with ring C to form still a fourth benzene ring. The name of the compound is derived from the steroid ring numbering convention (see page 117) which places the methyl substituent found on carbon 20. Methylcholanthrene can be prepared in the laboratory from desoxycholic acid, one of the bile acids.

Heterocyclic analogs of the polycyclic hydrocarbons may also be carcinogenic. The nitrogenous compounds 3,4,5,6 dibenzcarbazole (formula VI) and 1,2,5,6-dibenzacridine (formula VII) are examples. These carcinogens are water insoluble and are either applied to the skin as solutions in benzene or other fat solvents, inducing carcinomas, or are injected sub-



VI 3,4,5,6 Dibenzcarbazole



VII 1,2,5,6 Dibenzacridine

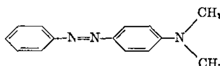
cutaneously as solutions in more physiological solvents such as lard or sesame oil, inducing sarcomas. It must not be thought that cancer follows the touch of these compounds unfailingly or quickly. Applications must be continued, usually over a period of months, for the effect to take place.

The carcinogenic activity of a given compound varies with the size and frequency of the dose, the manner of application (whether painted or injected), the solvent used, the presence of additional substances not in themselves carcinogenic, and the nature of the animal's diet. Thus 10-methyl 1,2 benzanthracene is highly active when administered subcutaneously, but only weakly active when applied to the skin, while the reverse is true of 2 methyl 3,4 benzanthracene. Although 1,2 benzanthracene *is not carcinogenic when applied externally or injected subcutaneously* it will cause hepatomas in some rats if included in their diet. Again methylcholanthrene in acetone is a more effective carcinogen than when dissolved in benzene, while in solution in anhydrous lanolin carcinogenic power is *almost entirely lost. The application of croton* oil to areas of skin being painted with 3,4 benzpyrene hastens the development of cancer, while the

simultaneous use of brombenzene or unsaturated dibasic acids with that carcinogen retards the effect A high fat diet often facilitates artificial induction of cancers while a diet low in the sulfur containing amino acids may retard the development of some kinds of induced cancers

Studies of carcinogens have revealed a form of variation of response more intriguing and perhaps more important theoretically than any of those listed so far When carefully inbred strains of mice were used as subjects for applications of carcinogens it was found that different strains varied in the frequency and rapidity with which individual members developed cancers of certain types These strains usually showed a higher incidence of spontaneous cancers of those types as well The use of animal strains of known cancer forming characteristics is thus highly important if significant results are expected in laboratory investigations

Micro organisms exposed to dilute solutions of a carcinogen such as methylcholanthrene exhibit an accelerated mitotic cycle (34) On the other hand yeasts exposed to camphor which is not ordinarily considered a



VIII p D methylaminoazobenzene

carcinogen show inhibited mitoses but continue growth to produce larger than normal cells which display morphological changes characteristic of cancer cells (37) Whether such cancer like phenomena among micro organisms are truly analogous to cancer in the multicellular organism remains of course a matter for speculation such experiments seem to show cancer as a truly basic cell disease

Other chemicals A chemical carcinogen second only to methyl cholanthrene in experimental usefulness is *p-dimethylaminoazobenzene* (formula VIII) a representative of a group of carcinogens completely different from the polycyclic hydrocarbons p Dimethylaminoazobenzene induced hepatomas in rats (but not in mice) when included in the diet The nature of the remainder of the diet affected tumor incidence carrots and rice stimulating the carcinogenic effect and liver depressing it This carcinogen is also frequently known as *butter yellow*

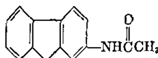
A related carcinogen of great versatility is *o-aminoazotoluene* which not only induces hepatomas on feeding but also results in various forms of cancer when applied externally or injected subcutaneously It is effective on mice as well as on rats

A peculiar carcinogen chemically in a class by itself is *N acetyl 2 aminofluorene* (formula IX) It is active only when administered orally

leading to the conclusion that not itself but a product of its metabolism is the true carcinogen Furthermore on ingestion it gives rise to a large variety of tumors exceeding in versatility even o aminoazotoluene in th respect

Radiation As in the case of chemical carcinogens the ill effects of short wave radiation was first discovered by tragic experience among the p oneer workers with x rays and radioactive materials Ultraviolet radiation is the least energetic radiation definitely known to be carcinogenic Exposure to ultraviolet was first shown to cause skin cancer in mice in 1928 (11) Rats and mice are both susceptible to ultraviolet albino varieties forming cancers more readily The induced cancers thus formed are in the derm s (i e sarcomas) whereas human cancers attributed to the effect of ultra violet are in the epidermis (carcinomas) The difference is thought to be due to the variation in the resistance of the skin to penetration by the radiation The wave length of maximum carcinogenicity of ultraviolet light is from 260 to 340 millimicrons

Cancer caused by radiation more penetrating than that of ultraviolet



IX N Acetyl 2 aminofluorene

is more apt to affect the blood and bones Leukemia and osteogenic sarcoma are frequently the result of continued exposure to x rays or gamma rays Radium is a special case Since it is similar chemically to calcium it is deposited in the bones if accidentally ingested Even small quantities so deposited may induce osteogenic sarcoma as a result of the localized rad a tion This was formerly a serious hazard among workers in industries using luminous paints involving radioactive substances

It is obvious that the importance of carcinogenic factors such as these is increasing rapidly in the modern world as a result of prospects for both atomic warfare and atomic powered industry

Physiological Carcinogens

Those cancer producing agents here termed physiological are similar in nature to those already discussed They differ in that the body in the course of normal living is exposed continuously or intermittently to their effects This means that although they are in general weaker carcinogens than many of those available in the laboratory, they are more important in a practical sense

Hormones There is a relatively high incidence of human cancer in the sex and accessory sex tissues In males this expresses itself as cancer of

the prostate, in females it is expressed as cancer of the breast and ovaries These tissues are remarkable in that they go through a period of sudden and rapid development during puberty under the influence of the sex hormones Now, the function of the sex hormones is primarily that of stimulating protein anabolism in these tissues (testosterone for instance, is the most potent stimulant of protein anabolism known) so that their normal function may be considered that of promoting growth Further more the sex hormones are steroids and thus bear some chemical similarity to the polycyclic hydrocarbon carcinogens and, in particular, to methyl cholanthrene It is not unreasonable to suppose, therefore that the hormones or some metabolic product of the hormones occasionally encourage growth somewhat too enthusiastically and bring about the non stop growth we know as cancer

The estrogens, which have been studied most in this connection, were found to be ineffective as carcinogens when smeared upon the skin of mice On the other hand, they were found to induce mammary cancer as well as cancer involving the uterus when injected into female mice (26) The success with which such cancers were stimulated depended to a very great extent upon the breed of mouse so that one might suppose a genetic factor to be involved Male mice, particularly of the cancer prone strains, were also affected by estrogen injection, developing mammary cancers and, in some cases cancer of the testes Synthetic estrogens, such as diethylstil bestrol, are as effective as carcinogens as are the natural hormones Unlike the natural hormones, diethylstilbestrol is effective when taken orally

All of these results, it should be emphasized, were obtained with mice Rats have lower incidences of spontaneous mammary tumors and are much more resistant to estrogen injection Rabbits, dogs and monkeys are extremely resistant to cancer induction by estrogen injection

The induction of tumors by hormones other than the steroid sex hor mones is less well known Various pituitary hormones, through their control of the production of the steroidal hormones by the adrenal cortex as well as by the gonads, may play important parts (12)

Diet and environment. No specific diet or food factor or combination of food factors have been found to be carcinogenic There is some reason to think, however, that excessive caloric intake may be correlated with a somewhat increased cancer incidence Strong (36) points out that man and domestic animals are more cancer prone than are wild animals and suggests that one possible explanation for it is that man and domestic animals tend to eat more than do wild animals

The effect of specialized environments upon human beings is extremely important in cancer production and was certainly the first cancer 'cause" noted by investigators In the 18th century, it was noticed that chimney

sweeps were more prone than the general population to cancer of the scrotum Workers in industries using coal tar more frequently developed cancer of the skin Similarly, cancers of the bladder are unusually common among workers in aniline dye industries In each case, the long continued effect of a carcinogen in the soot, coal tar, or aniline dye is to be suspected Much of the same may be said of people who are exposed, in the course of their work, to the effects of x radiation and gamma radiation Among them there is a definitely higher incidence of leukemia and osteogenic sarcomas This is of particular interest to the physician for personal reasons The incidence of leukemia among radiologists is ten times greater than among medical practitioners in other fields, while among physicians as a group it is 1 7 times greater than in the general population (20) A short wave radiation to which all humans are more or less subject is of course, the ultraviolet of sunlight It is an accepted belief that excessive exposure to the sun may result in a higher incidence of skin cancer (27)

Viruses

None of the carcinogenic agents hitherto discussed can be considered the primary cause of cancer They are too varying in nature and, probably, too indirect in their action It is more reasonable to suppose that their common action is to induce some change in some cell component or components and that this changed component is the true fundamental carcinogen Suspicion as to the nature of the cell component which undergoes this change falls most readily upon the nucleoproteins

First, nucleoproteins are capable of autoreproduction and are thought to control the chemical characteristics of the individual cell (see Chapter 7) which means that a cancer cell, with its changed chemical nature and its capacity to maintain its cancerous nature during reproduction, must possess a nucleoprotein or nucleoproteins differing from its normal ancestors The fact that strains of mice can be bred which are more susceptible to cancer, or less so, than the general population indicates that there are genic differences such that the nucleoproteins of one strain may be more readily changed to the cancerous variety by the action of particular carcinogens than those of the other The fact that ultraviolet radiation is carcinogenic may be significant in view of the fact that the purine and pyrimidine rings of the nucleic acids are about the strongest absorbers of ultraviolet in the body, while the effect of the more energetic radiations such as x rays may well be secondary in nature working through the formation of short lived free radicals which in turn, affect the nucleoproteins There is also reason to think that the chemical carcinogens affect nucleoproteins as we shall see

With all this in mind it is reasonable to suppose that it might be possible

to extract from cancerous material suspensions of changed nucleoprotein which on injection into healthy tissue would be capable of inducing the cancerous change there as well. Such suspensions have indeed been found and because of their resemblance to viruses both in size and in chemical nature as nucleoproteins have been termed *tumor viruses*.

The first such cell-free cancer-inducing agent of biological origin was discovered in 1911 by Peyton Rous. He found that extracts of spontaneous chicken sarcomas, when injected intramuscularly into other chickens, gave rise to sarcoma in the new hosts at the site of injection in about ten days. The properties of the extract indicate the active agent to be a pentose nucleoprotein in nature. A pentosenucleoprotein was isolated from chicken tumor I by differential centrifugation (7). When partially purified, this was shown to be capable of inducing tumor in another chicken in doses as low as 4×10^{-11} grams dry weight. Lipid is associated with the nucleoprotein and its removal renders the complex non-carcinogenic, although the lipid itself is not a carcinogen. The tumor virus is rather species specific, thus indicating that the nucleoprotein complex will operate only in cells of approximately the chemical nature to which it is accustomed. In general, only the tissue from which it has been extracted will be affected in a new host. Chicken tumor virus acts only with difficulty in such allied fowls as ducks, turkeys, and guinea hens (9). It must be injected in large amounts and within a short period after the fowl hatches. Similar tumor viruses have been found for spontaneous papillomas of the wild cottontail rabbit, except that here a desoxypentosenucleoprotein is present (2).

It was observed (3) that the offspring of a mouse belonging to a strain in which there was a high incidence of spontaneous mammary tumor did *not* tend to develop the tumor if the young was not allowed to suckle its mother but was nourished on the milk of a foster mother belonging to a strain with low incidence of mammary cancer. The reverse is also true. A newborn mouse which would ordinarily be expected, for hereditary reasons, to be free of mammary cancer will with high probability, develop this cancer if fed on the milk of a cancer-prone mother. This indicates the presence of a *milk factor* or mammary tumor inciter in the mouse. Virus-like particles have indeed been located in the milk from mice of cancer-prone strains (14) which were absent in milk from cancer-resistant strains. These particles were found to contain an as yet uncharacterized nucleic acid.

Theories of Carcinogenesis

There is a growing tendency to accept the supposition that carcinogenesis is the result of the presence within the cell of an abnormal nucleoprotein. The quarrel now is whether the abnormal nucleoprotein is exogenous or endogenous—that is, whether it is a body foreign in all its stages to the

normal cell or a body produced from a normal constituent of that cell To those who hold the former view cancer is a virus disease which is fundamentally similar to other virus diseases To account for the fact that cancer strikes in so erratic a fashion and is, as far as we know not contagious it is sometimes postulated that the cancer virus infects all cells and only becomes virulent in relatively rare cases where the cell metabolic processes are disturbed by the various carcinogenic agents An example of such a theory is that of Altenburge (1) *Those who believe in the endogenous* origin of the abnormal nucleoprotein consider the cancer cell to be the result of a mutation

Cancer and mutations A mutation may be looked on as the result of an imperfect self duplication of a gene In random changes most mutated genes would be lethals or at least would cause the loss of some characteristic previously present This has been demonstrated over and over again in such work as that on Neurospora (see page 58) If the gene controlling an enzyme synthesis were changed by mutation in such a direction as to cause growth without limit cancer would result

Spontaneous carcinogenesis would then be the result of a change in *nucleoprotein structure on the random basis that one in so many* gene autoreproductions is imperfect and one in so many imperfections is cancerous Naturally on such a basis the incidence of cancer would increase with age as it does since the older an organism is the more autoreproductions have taken place and the greater the chance for the cancerous *mutation* The incidence of cancer would also increase where more mitoses take place in a given time as in gonadal tissues at various stages in the sexual cycle

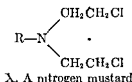
The incidence of cancer would also be increased if autoreproduction of nucleoproteins were interfered with so as to augment the chance of imperfect self duplication Any damage to the gene would obviously interfere with its capacity to duplicate its old self and damage to nucleic acids is known to result from the impact of high energy radiation (31) Increasing imperfections of hormonal control as the climacteric is approached and achieved may account for cancer of the breast or prostate in later life

The effect of chemical carcinogens is particularly illuminating It has been found that certain chemicals by their interference with the mitotic processes encourage an increase in the number of mutations An example of this is the effect of nitrogen mustards (formula X) upon the spores of *Penicillium notatum* (35) Such compounds are termed *mutagens* Mutagens cause symptoms *in vivo* much like those of overexposure to x rays both agents causing inhibition of mitosis injury to intestinal epithelium damage to hematopoietic and lymphoid tissue and necrosis of skin Among the mutagens so far found are various polycyclic carcinogens notably 20

methylcholanthrene 2 4 benzpyrene and 1 2 5 6 bidenzanthracene It has not yet been shown that all carcinogens are mutagens and *vice versa* but certainly the relationship has significance It is quite reasonable to suppose that the mutations induced by various mutagens are not always in the direction of the cancerous change and that mutagens may vary in their efficiency as carcinogens

Considering the somewhat limited state of our knowledge concerning the fine structure of nucleic acids and proteins in general, it is perhaps too sanguine to expect the elucidation of the chemistry of the cancerous change in the near future At least one method of attack is the determination of the distribution of purines and pyrimidines in the nucleic acids of cancerous and non cancerous tissues This was done in the case of a single human liver carcinoma (5) The adenine guanine ratios for both it and normal liver were found to be 1 5

Cytoplasmic factors The cell changes discussed in the previous section involve genes which, being located in the nucleus, may therefore be con



X. A nitrogen mustard

sidered organelles which are relatively well protected against environmental influences The mitochondria, organelles of the cytoplasm which contain nucleic acid are more exposed and presumably more vulnerable Furthermore the most general change in the enzyme patterns of cancerous cells involves the decrease in concentration of the enzymes of aerobic oxidation which are located within the mitochondria (and chicken sarcoma virus particles contain PNA characteristic of cytoplasm, rather than DNA, characteristic of nuclei) It is not beyond possibility therefore that the cancerous change may involve not the DNA of the gene but the PNA of the mitochondrion and that a decreased effectiveness in the biosynthesis of the cytochromes the flavo enzymes and the pyridino-enzymes may be one of the immediate results of the change

This would imply however that such cytoplasmic particulates would be capable of maintaining their new cancerous identity as they themselves autoreproduced and as the cell multiplied, despite the lack of change in the chromosomal gene make up Whether such autonomy on the part of cytoplasmic particulates is possible is a matter of much controversy (32) Those who believe that the cytoplasm can play a role in determining hereditary traits give those cytoplasmic factors the name of *plasmagenes*

One significant line of investigation in this respect involves certain strains

of *Paramecium aurelia*, whose presence in a culture was found to be poisonous to other strains (30) These 'killer" strains accomplish this effect by the production and liberation of paramecin an antibiotic which proves to be a desoxypentosenucleoprotein (38) Paramecin is produced by cytoplasmic particulates ('kappa"-particles) which apparently multiply at rates independent of that of the cell itself If the cell is in a medium under which conditions of rapid growth are possible, cell growth and multiplication outstrip kappa particle duplication, so that each generation has less paramecin producing material and is consequently less virulent as a killer Eventually, a cell is produced without any kappa particles and a new "sensitive" strain results despite the fact that the genetic consitution is precisely the same as it was in the original killer strain If at any time short of the complete loss of kappa particles the micro organism is placed in a medium where growth proceeds only slowly, kappa particles duplicate at a rate faster than cell division so that each cell generation becomes more virulent This represents the one definitely known case in the animal kingdom for cytoplasmic heredity independent of nuclear mechanisms

Enzymatic theories Regardless of the nature of the changed nucleo

As has been stated, the only such change consistent in cancer cells is the deficiency of the enzymes involved in aerobic respiration

How this respiratory enzyme deficiency affects protein anabolism is, as in so many aspects of the biochemistry of cancer, a matter of controversy One theory, advanced by Potter (29), postulates the existence of a competitive inhibition between a normal enzyme and a changed enzyme characteristic of cancer cells Both of these are produced independently, either through autoreproduction or through biosynthesis by a parent molecule, itself autoreproducing Potter believes that the normal enzyme is one which is essential to the respiratory chain and that in its presence growth is inhibited because the building blocks necessary for protein anabolism are utilized in respiratory processes The changed enzyme (enzyme X') competes for those same building blocks which it treats in a manner that does not interfere with their eventual use in protein anabolism The cell would turn to other methods of obtaining energy, such as glycolysis allowing protein anabolism to proceed unimpeded In the work cited, Potter suggests succinic acid dehydrogenase as the normal enzyme inhibited by "enzyme X"

CARCINOTHERAPY

The definitive methods of fighting cancer—extirpating it through surgery or destroying it by radiation—are of course beyond the scope of this

book. The search for biophysical or biochemical alternatives to surgery is unflagging because many cancers are unfortunately, through their position or their metastatic state, completely inoperable.

Nutrition

Since cancer incidence shows a positive correlation with a state of good nourishment and since a cancer in its continuous growth would be expected to require a steady supply of metabolites, attempts have been made to control tumor growth by decreasing over all food intake or by sharply restricting one or more food factors. Unfortunately, no method of dietotherapy has succeeded in doing more than delaying, not abolishing, the appearance of cancers in experimental animals, and none has succeeded in more than slowing, not stopping, the growth of cancerous masses.

Even these partial advantages are gained through diets so inadequate as to interfere seriously with the normal functioning of the body. Mice fed upon a calorie-restricted or cystine-deficient diet show decreases in incidence of mammary cancer as compared to well-nourished controls, but they also show irregularity in estrus, in some cases being completely anestrous, while mammary tissue failed to grow in the virgin and atrophied in the breeding female (39). Once a cancer is established, its growth can be slowed by drastic restriction of calories or of such food factors as riboflavin or pantothenic acid. In each case, however, body weight falls and the weight of the cancer grows larger in proportion. In general, cancers, being under no obligation to control or regulate their own growth for the good of the body as a whole, will continue to grow however restricted the host's diet. If no other source of energy or food factor exists, there are always normal tissues to be stripped so that in the end the host suffers more than the cancer. With respect to moderately restricted diets over prolonged periods as a general means of cancer prophylaxis, Greenstein says—

> When the vagaries of human nature, the possible carcinogenic hazards to which the individual is exposed and the demands of modern human civilization are all taken into consideration, it would be impracticable, not to say absurd, to suggest the prolonged abjuration of the few pleasures which life grants in the hope of avoiding cancer at some unknown and distant future. (15)

Radioactive Isotopes

Since the development of the cyclotron and particularly of the atomic pile, and the consequent mass production of artificial radioactive isotopes, clinical uses of these isotopes have increased tremendously. Radioactive isotopes, when introduced into the body, act as sources of penetrating radiation which in proper dosage may harm and even kill cells. This is ordinarily a most undesirable procedure. However, where the isotopes

can be so distributed in the body that the effect of the radiation is largely confined to cancer cells, the result may be beneficial.

The most successful such therapy involves the use of radioactive iodine in some cases of cancer of the thyroid. Iodine, after administration, is quickly and almost exclusively concentrated in the thyroid where the cancer cells then come under concentrated attack (along with normal cells, of course), while the rest of the body is relatively unaffected (33). Where the cancer has advanced to the point where thyroid tissue has lost the specialized ability to accumulate iodine, as is usually the case in thyroid metastases, the treatment becomes ineffective.

Unfortunately, no other element is concentrated by the body to the extent of iodine, so that there is no prospect as yet that isotope therapy will replace the classical x irradiation. Less marked concentrations are found in the case of radioactive strontium and radioactive phosphorus. The former localizes in the bones and may be helpful in the case of osteosarcomas. The latter is found in greater than average concentration in bone marrow, liver, spleen, and kidney. Since these tissues usually show the greatest infiltration of leukemic cells, radioactive phosphorus therapy is used in leukemia. Moreover, specialized techniques have been worked out in which injection of radioactive isotopes in non absorbable form near a cancer is employed. For a summary of such clinical devices in connection with cancer see Kamen (24).

Hormones

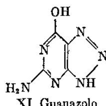
The effect of certain hormones on growth processes in the body leads to the hope that their use may serve to control the growth of cancer. The nearest approach to success has been achieved with the sex hormones. In cancer of the prostate any treatment which results in decreasing the effectiveness of male sex hormones tends to atrophy the prostate and cause the cancer to regress at least temporarily. Such treatment includes administration of estrogens and castration (21). The treatment is not always successful. For one thing, a later recurrence may take place due to compensatory production of male sex hormones by such steroid manufacturing glands as the adrenal cortex.

Chemotherapy

Gellhorn and Jones (13) summarize the state of the chemotherapeutic attack upon cancer up to 1949. The record is almost entirely one of very limited success. Urethane was found to produce temporary remission in chronic lymphatic and myelogenous leukemia, while nitrogen mustards could be used in disseminated malignant lymphomas. The authors con

clude however that of all the chemical compounds discussed none could in any way be considered a cancer cure and that in most cases such bene ficial effects as were observed turned out to be relatively transient

In 1949, the compound guanazolo (formula XI), similar to guanine except that carbon-8 was replaced by a nitrogen was found to stop the growth of cancers in mice The cancers did not regress and after cessation of therapy resumed growth (25) Any judgment of the value of this com pound or others like it upon human cancers must, of course await further experiments



XI Guanazolo

REFERENCES

1 Altenberg E The viroid theory in relation to plasmagenes viruses cancer, and plastids Am Naturalist 80 559-567 1946
2 Beard J W *et al* The nature of a virus associated with carcinoma in rabbits Surg Gynec Obst 74 509 1942
3 Bittner J J Some possible effects of nursing on the mammary gland tumor incidence in mice Science 84 162 1936
4 Black M M Biochemical studies in cancer diagnosis Dis Chest 16 169-176, 1949
5 Chargaff E *et al* Composition of human desoxypentose nucleic acid Nature 165 756-757 1950
6 Clark D G C *et al* Antiproteolytic activity of human serum with particular reference to its changes in the presence and considerations of its use for detec tion of malignant neoplasia Proc Soc Exper Biol & Med , 69 276-279, 1948
7 Claude A Particulate components of normal and tumor cells Science 91 77 78 1940
8 Coman D R Mechanism of the invasiveness of cancer Science 105 347 348 1947
9 Duran Reynals F The infection of turkeys and guinea fowls by the Rous sar coma virus and the accompanying variations of the virus Cancer Res 3 569-577 1943
10 Eriksen N *et al* Studies of various tests for malignant neoplastic diseases I The reduction of methylene blue by plasma J Nat Cancer Inst , 11 705-728 1951 II The Gruskin intradermal test *Ibid* 729-732 III The Hoff Schwartz intradermal test *Ibid* 733-738 IV The effect of zinc ion upon the serum alka line phosphatase activity *Ibid* 739-750 V The heat coagulation" of plasma *Ibid* 751-752
11 Findlay, G M Ultraviolet light and skin cancer Lancet 2 1070-1073, 1928

12 GARDNER, W U Hormonal imbalances in tumorigenesis Cancer Res, 8, 397-411, 1948
13 GELLHORN, A, AND JONES, L O Chemotherapy of malignant disease Am J Med, 6: 188-231, 1949
14 GRAFF, S, *et al* Isolation of mouse mammary carcinoma virus Cancer, 2 755-762, 1949
15 GREENSTEIN, J P *Biochemistry of Cancer* New York, N Y, Academic Press, 1947
16 GREENSTEIN, J P, AND JENRETTE, W V The liver catalase activity of tumor bearing rats and the effect of extirpation of the tumors J Nat Cancer Inst, 2: 283 291, 1941
17 GREENSTEIN, J P, AND LEUTHARDT, F M Enzymatic activity in primary and transplanted rat hepatomas J Nat Cancer Inst, 6 211-217, 1946
18 GREENSTEIN, J P, *et al* Chemical studies on human cancer I Cytochrome oxidase, cytochrome c, and copper in normal and neoplastic tissues J Nat Cancer Inst, 5: 55-76, 1944
19 GUTMAN, A B Serum "acid" phosphatase in patients with carcinoma of the prostate gland J A M A, 120: 1112-1116 1942
20 HENSHAW, P S, *et al* Incidence of leukemia in physicians J Nat Cancer Inst, 4: 339-346, 1944
21 HUGGINS, C Prostatic cancer treated by orchiectomy The five year results J A M A, 131: 576-581, 1946
22 HUGGINS, C Serum proteins in cancer Cancer Res, 9: 321-327, 1949
23 KAHLER, H, AND ROBERTSON, W v B Hydrogen ion concentration of normal liver and hepatic tumors J Nat Cancer Inst, 3: 495-501, 1943
24 KAMEN, M D *Radioactive Tracers in Biology* 2nd edit New York, N Y, Academic Press, 1951
25 KIDDER, G W, *et al* Purine metabolism in *Tetrahymena* and its relation to malignant cells in mice Science, 109. 511-514, 1949
26 LACASSAGNE, A Relationship of hormones and mammary adenocarcinoma in the mouse Am J Cancer, 37. 414-424, 1939
27 MOHS, F E Lack of extractable carcinogens in the skin of patients with multiple precancerous keratoses of actinic origin Cancer Res, 8 371-375, 1948
28 PFEIFFER, C A, AND ALLEN, E Attempts to produce cancer in Rhesus monkeys with carcinogenic hydrocarbons and estrogens Cancer Res, 8: 97-109, 1948
29 POTTER, V R Biological energy transformations and the cancer problem Advances Enzymol, 4. 201-256, 1944
30 PREER, J R A study of some properties of the cytoplasmic factor "Kappa" in Paramecium aurelia, variety 2 Genetics, 33: 349-404, 1948
31 SCHOLES, G, *et al* Action of x rays on nucleic acids Nature, 164 709-710, 1949
32 SCHULTZ, J The question of plasmagenes Science, 111: 403-407, 1950
33 SEIDLIN, S M, *et al* Spontaneous and experimentally induced uptake of radio active iodine in metastases from thyroid carcinoma A preliminary report J Clin Endocrinol, 8: 423-432, 1948
34 SPENCER, R R, AND MELROY, M B Effect of carcinogens on small free living organisms I Eberthella typhi J Nat Cancer Inst, 1. 129-134, 1940
35 STAHMANN, M A, AND STAUFFER, J F Induction of mutants in *Penicillium notatum* by methyl bis(β-chloroethyl) amine Science, 106. 35-36, 1947
36 STRONG, L C A new theory of mutation and the origin of cancer Yale J Biol & Med, 21: 293-299, 1949

37 THOMAS P T Experimental imitation of tumour conditions Nature 156 738 740 1945
38 VAN WAGTENDONK W J The action of enzymes on paramecia J Biol Chem 173 691 704 1948
39 VISSCHER M B et al The influence of caloric restriction upon the incidence of spontaneous mammary carcinoma in mice Surgery 11 48 55 1942
40 ZEIDMAN I Chemical factors in the mutual adhesiveness of epithelial cells Cancer Res 7 386 389 1947

CHAPTER 9

Reproduction and Heredity

All living organisms may produce offspring which in many ways duplicate the chemical make up of their parents, in this process chemical phenomena are involved The chemistry of reproduction is far from being completely understood Certain of the facts of heredity which can not yet be fully explained chemically seem to be potentially capable of a chemical interpretation, and this is briefly discussed here We also present the bare outline of formal genetics Furthermore, hereditary diseases are numerous, *and the wise physician will want to know something about the way in* which a child derives them from its immediate parents or remote ancestors We are going to assume that the physiology and cytology of reproduction are already known to the student, and only such portions of the cytologic process will be presented as seem essential to our argument

BIOCHEMISTRY OF REPRODUCTION

New human beings are begun when a single spermatozoon from a man reaches and fertilizes an ovum from a woman Fertilization takes place in the upper part of the fallopian tube Approximately three hours are required for the sperm to travel from the external os of the cervix to the site of fertilization The spermatozoa originate from the spermatogenic cells of the epithelial lining of the seminiferous tubules of the testicles, and undergo maturation during their passage through the tortuous 20 feet of tubule which makes up either of the epididymes In the acid environment of the epididymis the spermatozoa possess only minimal motility

Seminal Fluid

The seminal fluid or semen ejaculate consists of a suspension of spermatozoa in the *seminal plasma*, which is a mixture of the secretions of the epididymes, vasa deferentia, seminal vesicles, prostate, bulbo urethral (Cowper's) glands, and urethral glands (glands of Littré) Although there are 60 million or more spermatozoa per ml of ejaculate from the normal *fertile man, the actual volume of spermatozoa* plus epididymal secretion is less than 5 per cent of the total volume The volume of the ejaculate is usually between 2 and 6 ml, with the major portion contributed by the

secretions of the prostate and seminal vesicles The pH of fresh ejaculate is approximately that of blood plasma 7 4 but may increase to pH 8 with loss of CO_2 The pH of prostatic fluid is said by some to be highly alkaline but has been measured as about 6 4 (19) The chemical composition of prostatic fluid is given in table 12 Prostatic fluid does not coagulate on boiling It contains 255 to 1727 King and Armstrong units of acid phosphatase per ml (19a) Acid phosphatase can be found in voided male urine in much larger amounts than in the urine obtained by ureteral catheterization The difference is the contribution of the resting secretion of the prostate In health, very little acid phosphatase appears in the blood In cancer of the prostate, particularly if these are metastases, the acid phosphatase activity of the plasma is greatly increased This fact is of major importance in the diagnosis and clinical management of prostatic cancer

TABLE 12

Average composition of prostatic fluid (after Huggins)

	mEq/kgm H_2O	grams/100 ml
Na^+	156	0 36
K^+	30	0 12
Ca^{++}	30	0 06
Citric acid	156	1 0
Cl^-	38	0 14
Inorganic phosphate	1	0 003
Bicarbonate	8	0 07
Protein		2 5
H_2O	55 5	93–98

See also Hudson and Butler (18)

Traces of choline are detectable in fresh semen The concentration of choline increases with lapse of time after ejaculation, up to over 2 per cent *Phosphorylcholine* is the precursor, formed in the seminal vesicles, and hydrolyzed by phosphatases of the prostatic secretion (27)

Human semen coagulates promptly after ejaculation The clot liquefies in about 15 minutes The protein which clots is a product of the seminal vesicles The proteolytic enzyme which dissolves the clot has been identified in human prostatic secretion Motility of spermatozoa is limited during the clotted stage and is restored with liquefaction

In seminal plasma, or in certain artificial media such as buffered egg yolk, spermatozoa will retain their fertilizing power for considerable time if stored at temperatures just above freezing The secretions of the vagina are acid (pH 2 8 to 5 0), and spermatozoa do not survive well in an acid medium It is therefore believed that in a majority of cases insemination does not occur unless the spermatozoa are ejaculated directly against the

external os of the cervix Men and women vary in fertility, however, and pregnancy has been known to result when this condition was not fulfilled The high buffer capacity of semen presumably helps compensate for vaginal acidity The acidity of the human vaginal secretion is the result of the presence of lactic acid This acid is produced by lactobacilli the activity of which appears to be favored by the presence of estrogens.

Meaker and Glaser found the pH of the cervical secretion to be from 8 0 to 9 0, being above 8 5 in 80 out of the 100 cases examined by them The alkalinity of this secretion was not notably influenced by age, parity, menstrual cycle, endocervicitis, or viscosity of the endocervical mucus Others however have reported a cyclic production of a cervical mucus at about midcycle, which renders the cervix penetrable by spermatozoa for 4 to 10 days If this is so, it may be the basis of the so called 'safe period during other parts of the cycle, but it has been observed that impregnation can occur at any time What is still in doubt is the relative frequency of conception during different parts of the cycle The hormones involved in the production of spermatozoa and in the menstrual cycle are described in Chapter 6

The human egg, like other cells, consists primarily of a denser mass composed in great part of desoxypentose nucleoproteins, lipoproteins, phospholipids, and small amounts of pentose nucleoproteins, this mass is the nucleus and is surrounded by a less dense mass of protoplasm the cytoplasm The nucleus of the cell contains a certain number of bodies which are called chromosomes, which can be observed when the nucleus is in the process of division The numbers of chromosomes vary in mature cells from three pairs in certain plants and some species of Drosophila to more than one hundred in certain moths, crayfish, and some plants In man there are 24 pairs of chromosomes in the body cells In the formation of the gametes the number of chromosomes is reduced to half

Fertilization

Hyaluronidase. The human ovum is surrounded by a mass of ovarian follicular cells, the *cumulus oophorus* The compact innermost portion of the cumulus is the *corona radiata*, the cells of which are presumed to have a nutritive function for the ovum In the rabbit, several ova are bound together in a clot of cumulus cells and mucus The mucus clot can be dissolved *in vitro* by the enzyme hyaluronidase, or by spermatozoa There is hyaluronidase in the sperm of man, rabbit, and most mammalian species Hyaluronidase will not, however, bring about the detachment of the cells making up the corona, which are removed by a substance contributed by the fallopian tube The presence of cumulus cell masses has been con

sidered a barrier to the entry of sperm into ova, but instances of sperm entry into rabbit ova have been observed with the cumulus mass still attached (Chang and Pincus, 1951) Clinical reports have been conflicting on the improvement of human fertility by the use of bovine hyaluronidase as an adjuvant to human sperm, although it seems reasonable to suppose the hyaluronidase of the spermatozoa to be an important part of their mechanism for the penetration of the ovum There is no hyaluronidase in the ejaculate in cases where no spermatozoa are produced (12)

Parthenogenesis The development of an unfertilized ovum into a viable organism is a well recognized occurrence in certain non mammalian species Mammalian ova have repeatedly been activated by high or low temperatures, by chemical reagents, or by foreign sperm Such activation may continue *in vitro* through several stages of division Activated rabbit ova have been placed surgically into the fallopian tubes of pseudopregnant rabbits, and rabbit ova have been activated by low temperature while in the tube A small proportion of such experiments have resulted in the production of living young at term (7a)

Fertilizin. It was demonstrated in 1913 (25) that waters which had been in contact with eggs of the sea urchin or certain other species were able to cause the agglutination of the homologous spermatozoa and Lilly coined the term fertilizin for the active agent in this egg water Since then fertilizin has been obtained from eggs of various species of invertebrates, and has also been reported to occur in eggs of vertebrates The fertilizins seem to originate from the gelatinous coat of the egg which slowly goes into solution as the egg stands in water There is some evidence that this agglutinating activity of fertilizin on the spermatozoa has some connection with the fertilization process perhaps serving to cause the spermatozoon to stick to the gelatinous surface of the egg from this point it can begin its process of boring into the egg to reach the nucleus Fertilizin has been obtained in electrophoretically homogeneous form from certain eggs and some of its chemical properties have been studied It is highly acidic, contains about 25 per cent of sulfate or a sulfuric ester such as is found in chrondroitin sulfate or mucoitin sulfate The preparation gives both protein and polysaccharide reactions and could be considered as belonging to the group of mucoproteins However, according to Tyler (43), it is not possible to separate it into a protein and a polysaccharide constituent He believes this is because the various sugars and amino acids are interlinked, forming a compound which is neither a protein nor a polysaccharide He considers fertilizin to belong to the group of compounds which include the human blood group substances Fertilizin was found to have a minimum molecular weight of 82 000

Amniotic Fluid

The amniotic fluid is considered to be composed of extracellular fluid of both fetal and maternal origin, to which is added hypotonic fetal urine. *It is in equilibrium with the maternal and fetal blood plasma*, and contains the usual inorganic ions found in extracellular fluid in approximately the same concentrations (see Chapter 15). Highly variable protein levels have been reported, ranging from 0.2 to 1.5 per cent. The glucose is lower than that of blood plasma, usually about half the blood value. The uric acid concentration is variable but usually higher than that of the maternal blood. The volume of amniotic fluid at term is usually between 0.5 and 1.3 liters.

CELL DIVISION

Chromosomes

When a cell divides, two descendant cells are produced, each of which derives its nucleus and its cytoplasm from the nucleus and cytoplasm of the mother cell. The division of the cytoplasm is relatively simple. A furrow appears on the surface of the cell around its whole circumference. This *furrow gradually cuts deeper and deeper into the cell until it separates the* cytoplasm into two halves.

While the cytoplasm is dividing, the nucleus is also dividing involving a rather more elaborate series of events. The nucleus in the resting cell seems to be a relatively undifferentiated vesicle, but during division well defined structures become visible. They may have varied shapes, they may be round, or short rods, or long rods, or they may be V-shaped or J shaped. These structures pick up certain stains more intensely than the rest of the and they are therefore called chror *and "body"*

After chr the nuclear membrane disappears and the nucleus as a separate entity is gone. The chromosomes are associated with a structure called the spindle which develops inside of the cell. By the time the spindle is fully formed, the chromosomes all appear double, the two identical parts lying side by side but separated by a clear space except for being joined in one short region. Finally the pairs of chromosomes separate, one member going into one half of the dividing cell and one member into the other. Thus the divided cells each have a complete set of chromosomes, which results from this division. During this stage of division the chromosomes have a large amount of the highly stainable desoxypentose nucleoprotein, and we have reason to believe that this nucleoprotein is of the highest importance in the process of heredity. In some way the chromosomes of the original cells have managed

to duplicate themselves by building a copy out of the surrounding protoplasm of the cell

Chromosome Components.

Chromosomes, as they are visible during cell division have been shown to be coiled like spiral springs The relatively slender threads of which these are formed are, in many cases, too narrow to be resolved by ordinary microscopic methods When these threads can be seen, however, they appear



FIG 7 Chromomere pattern of a strand of a typical chromosome

to be covered by a succession of fine beads called *chromomeres* Chromomeres are of different sizes at different points, and their arrangement is characteristic for each chromosome The pattern enables the chromosome to be identified For instance, one chromosome might have two successive medium sized chromomeres at one end, then a larger one, then a series of three very small ones This is shown in the diagram of a specific human chromosome (fig 7)

From the diagram one also sees that the distances between the chromomeres may vary as well as their size and staining capacities

A better idea of the linear arrangement of the chromosomal elements is found by study of the giant chromosomes from the salivary glands of the

larvae of certain species of flies Here the chromosome, instead of appearing as a slender thread, looks like a rather wide cylinder marked by large numbers of cross bands or discs It has been suggested that these giant chromosomes are the result of the lining up of large numbers of chromosome threads in parallel

This linear arrangement and characteristic pattern of the cross bands in the giant chromosome or of the chromomeres in the threadlike single chromosomes is of great importance in connection with the linear arrangement which must be postulated to explain the known facts of genetics

GENETICS

It has been found that inheritance is particulate in nature, that is, characteristics from mother and father do not blend in the germ plasm (although in some cases they appear to blend in the offspring), but remain separate and may segregate again in the offspring of the next generation Our knowledge of inheritance in an exact sense all goes back to the experiments of the Austrian monk, Gregor Mendel, who first worked out the essential laws by experiments with the garden pea Mendel's work made no impression on his contemporaries and the publication of it in 1866 in the Proceedings of the Natural History Society of Brunn attracted no attention despite the fact that copies of this journal reached various parts of the world including the United States Nevertheless the work was so carefully and brilliantly conceived that when it was rediscovered at the beginning of this century by Correns De Vries and von Tschermak (42), it was found to explain the mechanism of inheritance—not only in the garden pea but in all plants and animals, including man, which have subsequently been studied [1]

Mendel's Laws

Particulate Nature of Inheritance.

Dominance Among hybrid or crossbred offspring each individual exhibits just one of each pair of contrasted ancestral characters, to the total (or almost total) exclusion of the other Intermediate forms do not appear

Mendel called the character that prevailed dominant, and the character that was suppressed (or apparently suppressed) *recessive* Thus the first important result was the discovery that, *dealing with pure lines* crosses

between a plant with the dominant character and a plant with the recessive character yielded offspring which, as regards the character in question, all resembled the dominant parent If we designate the appearance of the parents as D (dominant) and R (recessive), Mendel's first result may therefore be expressed thus The cross Dominant by Recessive, produces Dominant appearing offspring Or in symbols—

$$D \times R = D$$

Later work has shown that *complete dominance* is much less common than was originally thought In fact, some effects of each gene of the pair are generally discernible in the hybrids (5)

In the next generation the crossbred plants which had been produced by crossing D and R and which were all apparently like D, were allowed to fertilize themselves, and it was then found that their offspring exhibited both of the two original forms, showing on the average three D's to one R We may take as an example the tall x dwarf cross 1064 second generation plants were produced, 787 were tall (D) and 277 were dwarfs (R)

When any of these recovered" dwarfs (i e, recessive descendants of a group of plants all "Dominant" in appearance) were allowed to fertilize themselves they gave rise to dwarfs, R, only, a process which could be continued for any number of generations In other words, the recessive character bred true The hereditary unit tending to produce it (we call such units *genes*) had not been altered by its association in the hybrid with the dominant (D) gene for tallness

On the other hand, when the dominant appearing descendants were allowed to fertilize themselves, one third of them produced pure dominants which in subsequent generations, when allowed to fertilize themselves, gave rise to dominant descendants only, two thirds of them, however, were an impure group which produced once again the characteristic mixture of dominants and recessives in the proportion of 3·1 These results are shown in figure 8 The result of the initial hybridization is a first generation (F_1) which resembles the dominant parent They may be represented by the symbol (D), since they look like the dominant, even though they carry the possibility of producing offspring characterized by the recessive character—that is to say, the recessive character has remained latent in the inheritance

The genetic formulas of the various individuals are evidently as follows, where D represents the dominant gene and R the recessive

Type as identified by inspection	*Genetic type as identified by breeding experiments*
D	DD or DR
R	RR

From this it may be seen that the pure dominant individual, which possesses only D genes, can produce only D offspring when it fertilizes itself and R can produce only R The dominant appearing individuals (D), however, being genetically mixed, can produce D, (D) and R offspring in the ratio of 1:2:1

Independent assortment of unit characters. Mendel's second law states that each pair of hereditary factors showing this dominant recessive

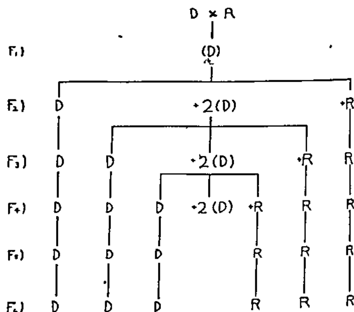


FIG 8 Diagram illustrating "Mendelian splitting" or segregation which occurs ... nd recessives breed true, but the ... continue ... mozygous

dominants

relationship behaves quite independently of every other pair This is called the law of independent assortment This second law is not without exceptions, for reasons which will presently appear However, it does hold for many factors, and unless the genes were connected together in some way in the organism, it would be expected to hold for all factors influencing inheritance Reproductive cells (gametes) having all possible combinations of genes would be expected The fact that the law does not always operate suggests that there may be some physical connection between certain genes, or in other words, the genes may be transmitted in groups during the process of reproduction

To take an actual instance of independent assortment Mendel found that the character pair yellow green (of which yellow is dominant) and the pair round wrinkled (of which round is dominant) assorted independently Either gene pair considered alone, in the F_2 generation produced dominants and recessives in the expected 3 1 ratio Mendel crossed a round yellow race with a wrinkled green one and, as expected got nothing *but round yellow seeds in the first* (F_1) *generation If such plants were* then crossed with plants which were genetically pure (*homozygous*) for both of the recessive genes in question (i e wrinkled green peas), there were four possible types of offspring, namely round yellow round green wrinkled yellow and wrinkled green, the genes, assorting independently, produced the four possible types of offspring in equal numbers

Mendel represented the dominant gene for round as *A* and the recessive gene for wrinkled as *a*, the dominant for yellow as *B*, and the recessive for green as *b* This would give us for his hybrid (heterozygous) pea, the

TABLE 13

Results of crossing AaBb with aabb (Mendel)

FEMALE GAMETES	MALE GAMETES			
	AB	*Ab*	*aB*	*ab*
ab	*AaBb*	*Aabb*	*aaBb*	*aabb*

formula *AaBb* The pure recessives would be *aabb* They could produce only one kind of gamete, namely, *ab* The heterozygotes could produce four kinds of gamete, in equal numbers, *AB* *Ab*, *aB*, and *ab* Combining these with the gametes of the wrinkled green peas, we get four possible combinations, and therefore *four possible types of offspring in equal numbers* namely, *AaBb*, *Aabb* *aaBb* and *aabb*, as shown in table 13 This was confirmed by experiment

If the reader has followed this reasoning, he should have no difficulty in predicting the outcome of a cross of two plants heterozygous for both genes (or, what amounts to the same thing the results of allowing a doubly heterozygous plant to fertilize itself) Each plant produces the four possible gametes, *AB*, *Ab*, *aB*, and *ab* in equal numbers It is a matter of chance which ovum is fertilized by which pollen grain, so we get all the possible combinations, as shown in table 14

Since round is dominant over wrinkled, and yellow is dominant over green, not all these sixteen different genetic types will be distinguishable in the offspring We find, in fact, that just four types can be distinguished, and they are classified as shown in table 15

It will be noted that not only are there just four distinguishable types of

offspring but since each type of gamete is produced as frequently as any other the four types of offspring will occur in the ratio shown which is 9 3 3 1 The actual ratio observed by Mendel (28) was 315 round and yellow 101 wrinkled and yellow 108 round and green and 32 wrinkled and green

The use of capital letters for the dominant gene and small letters for the recessive was continued by Mendel s successors but when it was found

TABLE 14

Combinations resulting from the cross of individuals hetero ygous for two characte s

FEMALE GAMETES	MALE GAMETES			
	AB	*Ab*	*aB*	*ab*
1*B*	*AABB*	*AABb*	*AaBB*	*AaBb*
Ab	1*ABb*	*AAbb*	*AaBb*	*Aabb*
aB	*AaBB*	*AaBb*	*aaBB*	*aaBb*
ab	*AaBb*	*Aabb*	*aaBb*	*aabb*

TABLE 15

Phenotyp cally d fferent offspring from the mating shown in table 14

AB (ROUND YELLOW)	*Ab* ROUND GREEN)	*aB* (WRINKLED YELLOW)	*ab* (WRINKLED, GREEN)
AABB	*AAbb*	*aaBB*	*aabb*
AABb	*Aabb*	*aaBb*	
AaBB	*Aabb*	*aaBb*	
AaBb			
AABb			
AaBb			
AaBB			
AaBb			
AaBb			

that sometimes there were more than two genes which could occur at a chromosome locus the system ceased to be so satisfactory

No Blending Inheritance

Inheritance is particulate in nature as we have seen in reviewing Mendel s work That is the germ plasm is passed from parent to offspring in the form of discrete particles and not as a portion of a more or less uniform mixture of the germ plasm of mother and father These units of heredity or of germ plasm are known as genes It is customary to cons der a characteristic such as blue eyes diabetes or hemophilia as due to the action of a single gene pair one member coming from the father and one

from the mother In some cases the father contributes no gene at that locus, so the action of the mother's gene, although it is recessive, is unhampered, It is probable that nearly every gene in the inherited constitution of the individual has *some* effect on nearly every character, although in some cases the effect must be slight

Genes seem to control all the vital processes of an organism, although some examples of "cytoplasmic' inheritance have been found to extend over a few generations in certain lower forms It has not been proved, and probably can never be proved, that all the characteristics of a given organism are determined by genes As has been pointed out (37), there is a reason why we shall never completely prove the genic nature of some of the really major characteristics of any form of life Since these major characteristics, such as having lungs a heart, blood, and so forth, are absolutely vital to the life of the organism, a change in the genes which control them is almost certain to lead to a condition which will interfere with normal development, or in other words will produce what geneticists call a *lethal* A lethal gene is a gene which kills the organism which inherits it It usually requires a double dose, since lethals are generally recessive It can be seen that dominant lethals could not persist beyond one generation Changes or mutations altering for the worse the absolutely essential features of an organism will be eliminated almost immediately by the action of natural selection, and each species will be homozygous for the normal (non lethal) aspect of genes of such major importance

The importance to biology of the concept of particulate inheritance can hardly be overestimated, but until the discoveries of Mendel and those who confirmed his work became known, it was assumed by practically all laymen, and by biologists too, even by Darwin, that inheritance was of a blending character For example, it was supposed that a parent with a black skin and a parent with a white skin would always produce children with brown skins, and that these brown children, mated with similar brown children, would of course have brown offspring, for it was thought that the two characters, or types of 'blood", were forever mixed, just as ink and milk poured together into the same container can never again be separated

When considerable numbers of genes are involved, as in skin color,[2] heredity does at first sight appear to be of a blending character Genetic analysis of such characters is difficult The genius of Mendel led him to select for study varieties which differed *from each other in only a few characters, genetically as well as apparently*, so that he *readily recovered the* parental types from crosses among the offspring All inherited characters which have been fully analyzed have always been found to depend on

[2] Davenport proposed a theory involving only two pairs of genes for skin color in man, more are probably involved however

particulate genes which retain their individuality even in hybrids This applies even to those apparent cases of blending inheritance which have been adequately investigated

We may discuss briefly an example which, without careful investigation might seem to be an example of the blending type of inheritance Among chickens there are a number of genetically pure color types, including the types black Minorcas and splashed white Plymouth Rocks, each of which breeds true But crossing black Andalusian fowl with splashed whites pro duces a slaty grey intermediate, the so called Blue Andalusians (1) It seems almost self evident to the uninstructed observer that the genetic material of the two parents has been blended in the offspring to produce an intermediate and novel type, but this is not true Breeding experiments reveal that "blending" is not the correct explanation Crosses of Blue Andalusians among themselves produce both black and splashed white offspring as well as the Blue Andalusian, and it is absolutely impossible to obtain a stock of Blue Andalusians which will breed true The two types *of hereditary material have not blended, but during a period of coexistence* in a certain individual they combine their effects to produce a new type of appearance The genetic materials however, have remained unchanged, *and when they again emerge by themselves in the offspring* they produce the same effects they produced in the original parents A cross of Blue Andalusians gives the expected 1 2 1 ratio

Since the Blue Andalusians do not look exactly like either the black or the splashed white parent, we miss in this case the complete dominance which Mendel observed in his peas There are many other examples Thus a cross between a "Chinese" primula which has wavy crenated petals and a "Star" primula with simply notched petals gives progeny intermediate between the two parents, and yet, as the next generation shows, the case is one of Mendelian inheritance—that is, the two characteristics have stayed distinct in the germ plasm and both parental types, as well as the mixed variety, appear among the offspring

In many cases the hybrid, while exhibiting on the whole the character of the dominant, may show also some influence of the recessive character but not enough to warrant our speaking of the result as a "blend" Thus, when white (dominant) Leghorn poultry are crossed with brown (reces sive) Leghorn, most of the offspring have some "ticks" of color When these are inbred they produce one quarter brown and three quarters pure white or white with a few "ticks"

When dealing with characters which can only be present or absent, it is often not possible to measure degrees of dominance, although in situations which at first seem to belong to this category (as in the case of the blood groups of man) some information as to degrees of dominance has been

obtained If we are dealing with quantitative characters (that is, characters which may be counted, measured, or in some way expressed in numerical terms) it is possible to express the degree of dominance more exactly The eye of the fruit fly Drosophila, is made up of a number of separate elements or facets The number of these elements in any individual eye can be counted and the effect of various genes on them can be determined Figure 9 shows the effect of a mutant gene which is called "Bar", when it is present in the homozygous and heterozygous condition and also shows the results of facet counts on the homozygous "wild" type without the mutant gene In this figure, the vertical axis represents the percentage

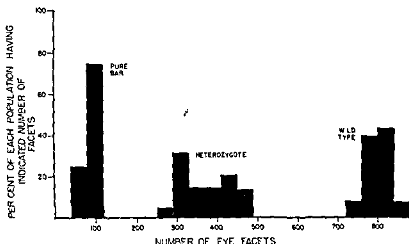


FIG 9 Quantitative effects of a gene

frequencies of each of the three types, and the horizontal axis, the facet numbers The three *genotypes*[3] are sharply distinguished although there is a certain normal variability within each type Dominance is clearly incomplete in this case, since the heterozygote is decidedly different from either of the homozygotes It has been suggested (44) that early in development a facet forming substance is produced in the young fly and that the Bar gene starts up a new chemical reaction which breaks down this substance In a later stage, the actual process of facet formation occurs, but the number of facets formed is determined by the amount of facet substance which still remains

[3] The *genotype* of an individual is a summary of his genetic constitution, the *phenotype* is a statement of his outward appearance, or what you can determine by direct tests

Blending inheritance seems superficially so logical that it was accepted almost without question even by such pioneers in biometry as Galton and Boas, although the latter abandoned it in his later papers But it is totally wrong, and as Fisher (10) has pointed out, almost the whole of the revolutionary effects of Mendelism can be seen to result from a knowledge of the particulate character of the hereditary elements Although Darwin accepted the fusion or blending theory of inheritance, just as other men of his time did, and although he almost certainly never heard of Mendel's work, it is interesting to note that he did at certain times feel the need for a non blending theory In a letter to Huxley which was probably dated 1857, these sentences occurred (10)

Approaching the subject from the side which attracts me most, that is inheritance I have lately inclined to speculate very crudely and indistinctly, that propagation by true fertilization will turn out to be a sort of mixture, and not true fusion, of two distinct individuals or rather of innumerable individuals as each parent has its parents and ancestors I can understand on no other view the way in which crossed forms go back to so large an extent to ancestral forms But all of this of course, is infinitely crude "

Fisher points out that this idea was never developed by Darwin, probably because of the rush of work preceding and following the publication of *The Origin of Species*

Chetverikov (8) and Fisher (10) have also pointed out that on the assumption of blending inheritance the heritable variability of the species is halved each generation It was realized even by Darwin that domesticated species displayed far too much variability for this to be true, and modern studies have shown that wild organisms exhibit about the same degree of variability as the domesticated The hypothesis of blending inheritance which at first sight seemed so reasonable, must now be completely rejected

Chromosomes as Grouped Genes

Mendel supposed that his hereditary factors were present in pairs in a mature organism, but single in the reproductive cells or gametes Soon after Mendel's work was rediscovered, it was noticed that there was a close parallel between this hypothetical situation and the actual position of the chromosomes

It had been observed that the pairs of chromosomes present in the cells of the mature organism separated themselves during the formation of gametes The chromosomes can not be identical with the hypothetical units of heredity of Mendel because there are not nearly enough of them in any organism to account for the great variety of the known genetic factors Furthermore, it is now known that the individual genes of any organism are transmitted in groups during the reproductive process, and

it is generally believed that the number of these groups is equal to the number of chromosome pairs Each group of genes within a particular chromosome then assorts independently of other groups (Mendel's second law) Members of any given group usually stay together

In the formation of reproductive cells (gametes), each pair of chromosomes splits up, and thus every gamete possesses one member of each pair In the process of gametogenesis the chromosomes are not simply separated into two numerically equal groups At least one member of each chromosome pair must be present in every adult cell for that cell to be able to develop and function normally Therefore every gamete will contain one chromosome from each pair

From the chromosomes of the gametes, combined after fertilization, appear new combinations of the various chromosomes Since genetic experiments have shown it to be purely a matter of chance which chromosome of each pair enters into any gamete, we are justified in stating that the chromosomes are shuffled and redealt in single sets The members of the different pairs recombine at random and all possible combinations can have equal probability Knowing the number of pairs of chromosomes in any given organism, we may compute the number of possible different combinations For two pairs we have 2^2 which means four possible combinations For three pairs we have 2^3 equaling 8 combinations, and for four pairs 2^4 equaling 16 combinations For n pairs 2^n gives the number of combinations

In man there are 48 chromosomes per adult cell, or 24 pairs, so the number of combinations is therefore 2^{24} This gives the staggering total of 16,777,216 possible combinations, of which only two are exactly the same as either original parental combination Therefore, the chances that a human being will repeat either of the parental combinations exactly is only one in 8,388,608

Genes

Dominants and alleles. From the foregoing, it should be clear that each organism inherits two genes affecting each of its various characteristics, one from the mother and one from the father, and that in respect to the more vital characteristics of the organism, in all probability these two genes are substantially identical Genes which affect minor external or less important characteristics may sometimes be different in the two parents For example, one parent may have had a particular shade of brown eyes and the other blue eyes, and the eye color in each case was determined by the presence of a gene, or combination of genes, capable of occupying a certain place or locus in the serial arrangement of genes on one particular chromosome

of time beginning long before the Bronze Age, say about 7000 B C, and extending up to the present time Pearl observed no changes in this particular Drosophila gene in any phase of the experiment

Multiple alleles. In Drosophila a series of genes has been observed, affecting eye color, which may produce colors ranging from the normal red of the Drosophila eye to coral, which is a very little lighter, to eosin, cherry, apricot, buff, tinged, and through shades of ivory to white

Where only two genes are available from any crossing, only three geneti- [illegible] each kind [illegible] when a number of genes form the series of allelomorphs, the possible types are greater (40), and the number of different genotypes with n allelomorphs is equal to $n\frac{(n+1)}{2}$ Thus the eight allelic Rh genes now known in man (page 392) makes up 36 different genotypes.

Among the allelomorphic gene series which are known, most members of the series affect the same characteristic of the organism But this does not necessarily have to be so In Drosophila, for instance, there is a gene which produces a disarrangement of the normal order of the rows of facets in the compound eye Another gene which can occupy the same locus in the chromosome seems to produce no effect on the eye but causes little [illegible] ch is heterozygous [illegible] ly and has neither [illegible]

[illegible] s all having some effect on the same characteristic enables us to interpret the so called continuous variability often observed in characters Thus, although it is comparatively easy to classify men into four different blood groups which do not overlap, it is not possible to classify mankind into sharply different groups in regard to height (aside from perhaps the Pygmies), and we observe all sorts of gradations between tall men and short men, although stature is partly determined by heredity To a certain extent skin color also furnishes an example of this type of continuous variability When cases of supposed continuous variability are thoroughly investigated genetically, however, they are found to be the effects of a considerable number of genes, acting generally two at a time, or in some cases acting several at a time, but modified by environment

Number and size of genes. Considering the minuteness of the head of the spermatozoon, which we know must contain at least one out of every pair of genes which the adult organism is going to receive (ignoring cases where the Y chromosome has certain genes missing), we can see that a

In practice, genetic linkage is detected by observing that the two genes are always associated in inheritance. At first sight such observation may seem impossible, since crossing-over has usually resulted in the production of all the different combinations of the two gene series. However, an inspection of the accompanying illustration (table 16) will show that, under ordinary circumstances, linkage of the two genes will be demonstrated by the fact that from any given combination of parents only certain of the theoretically possible types of offspring will be produced, whereas if the genes were on different chromosome pairs and thus assorted independently all the possible types would be produced with the theoretical frequencies.

A type of genetic linkage which is particularly easy to detect is sex

TABLE 16

Backcross demonstrating linkage in maize

S La/su la ♂ x su la su la ♀

EGGS	SPERMS (POLLEN)			
	S La	su la	(S la)	(su La)
su la	S La/su la 45.5%	su la/su la 45.5%	S la/su la 4.5%	su la/su la 4.5%

The symbol S stands for a dominant gene producing starchy kernels, su for a recessive producing sugary kernel. la stands for a recessive gene producing lazy sprawling type of growth of the stalks, La for its dominant normal allele. The types of sperm in parentheses are produced by crossing over. In the absence of crossing over linkage would be complete and only two of the four possible types of offspring would be produced. The inequality in frequencies of the various types is a measure of the degree of linkage.

linkage. This occurs when a gene is carried in one of the so called sex chromosomes. It will do no harm if we oversimplify the real situation a bit and state that in man, as in a number of other organisms, sex is determined by a particular one of the twenty four pairs of chromosomes. The female is produced from a fertilized ovum containing two such chromosomes, both of the same kind (called the X chromosomes), and the male is produced when the two members of the chromosome pair are not alike, one being the so-called Y chromosome. From data compiled on organisms that have been rather thoroughly studied, such as the fruit fly, it has been concluded that the Y chromosome, the presence of which determines maleness (or we might perhaps better say that it takes two X chromosomes to determine femaleness), contains very few genes of any sort. In fact it seems to be practically inert genetically. This means that genes in the X chromosome, even when they are recessive, will not have their expression repressed in the male, since there exist no corresponding genes in the Y chromosome which

is paired with the X chromosome, and thus there is nothing to repress their effect As an example of the effect of such a gene, we may mention the deficiency in clotting power of the blood which is called hemophilia An other example is the inheritance of "ordinary" red green color blindness

It seems clear that the gene for color blindness (of the sort to which we have referred) is in the X chromosome and that it is a recessive A woman who has only one such gene therefore will still have normal color vision, because of the dominant action of the normal gene in the other X chromosome, but a man having one such gene will be color blind because of the

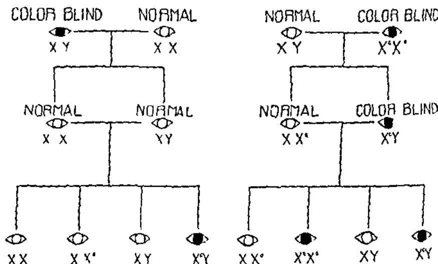


Fig 11 Diagrammatic representation of the supposed mode of inheritance of ordinary (red green) color blindness The symbol X indicates a normal X chromosome X' indicates an X chromosome which carries the gene for color blindness

absence of the dominant gene in the X chromosome, which would otherwise suppress the action of the color blind gene A mating between a color blind male and a homozygous normal woman would produce normal males and normal females It will be seen from figure 11, however, that the female offspring will carry in one of their X chromosomes color blind genes Consequently on the average half of their sons will be color blind Color blindness is fairly common in men running at least as high as 5 per cent in some populations but it is quite rare in women An inspection of the diagram will show that for a woman to be color blind it is necessary for her father to be color blind and for her mother either to carry the color blind gene in a single dose or to be herself color blind, which is of course much less likely

At first sight it might be thought that the existence of sex linkage disproves the earlier statement that linkage is not detected by association of two traits in the population, for there is unquestionably an association between maleness and color blindness in the population The point is that such association between maleness and color blindness is not necessarily due to sex linkage, but may be due to the effect of sex on the expression of an autosomal gene (i e , one not upon the X and Y chromosomes) If color blindness for example, were caused by an autosomal gene which was dominant in males but recessive in females, much the same picture of association would be produced And human traits have been studied, as for example baldness (16) and taste reaction to phenylthiocarbamide (7, 9), in which the expression of the gene seems to be better in one sex than in the other In the case of taste reaction to phenylthiocarbamide the gene seems to be expressed better in the female but in the case of baldness the gene seems better expressed in the male

Sex linked inheritance can be distinguished from autosomal sex limited or sex influenced inheritance only by the study of descendants of affected males (36) To determine whether an apparently sex linked character could reach full expression in females, homozygous individuals from matings between female carriers and affected males would have to be examined

More than twenty examples are known of genes which are apparently carried in the X chromosome of man It is clear that any two of these genes will be themselves genetically linked since they are carried on the same chromosome, and the study of human pedigree supports this idea (3)

Expressivity and penetrance. There are two terms applying to the manifestation of genes which we at times have occasion to use The first is called *expressivity* and is a measure of the amount of kind of effect shown in an individual possessing the gene The second is called *penetrance* and is frequently measured as the percentage of individuals who, when they possess the gene, show any effect from it Naturally, most of the genes which have been chosen for experimental work with the lower forms show a very high or complete penetrance The frequency with which the gene produces its effect depends both on the environment and on the genotype, the effectiveness in the heterozygous condition often being less A good example of environmental influence is found in the gene "giant" in Drosophila Here lack of sufficient food for the larvae results in fewer giants The expressivity of this gene is nearly uniform, all giants being about the same size Such genes would seldom be used in experimental work with lower forms In man, however, we can not pick and choose our material to the same extent, but must take it as it comes, and we shall have to deal with some genes which do not show complete penetrance In the case of

deleterious genes of course, natural selection would tend to postpone more and more the age of onset of the symptoms, possibly by the selection of suitable modifying genes

Mutation. In this book we shall restrict the term mutation to changes in the composition of individual genes The first mutation to be recorded in an animal appeared in 1791 in a male lamb belonging to the flock of Seth Wright, a Massachusetts farmer As a result of this mutation, the lamb had very short bowed legs, and a special breed was developed from it by deliberate selection because it was an advantage to farmers to have short legged sheep which were not able to jump the stone walls which surround the New England sheep pastures This early breed eventually became extinct, but the same mutation later appeared a second time this time in Norway, and the short legged breed was reconstructed Since that time numerous other mutations have been observed in animals and we have some reason to believe that we know the exact origin of one or more of the mutations since found in man A mutation of a normal to a hemophilia producing gene is thought by some (14) to have occurred in the person of Queen Victoria who transmitted it to many of her descendants including members of the Russian and Spanish royal families

The self propagation of genes is one of the most remarkable things about them Muller (31) has commented on the fact that not only is this self propagation in itself remarkable but the study of mutations reveals the still more remarkable fact that after mutation when the chemical structure of the gene has changed the gene still has the property of propagating itself, *its new self* Although a given gene may be changed in various ways there is generally a strong tendency for any given gene to undergo changes of some particular kind (32), so that it usually mutates in some one direction rather than in another Muller suggests that an animal is generally in such good equilibrium with its environment (as the result of countless generations of natural selection) that any change is likely to be a change for the worse How extensive the change within an individual gene has to be before we recognize it as a mutation is still unknown Muller thinks however, that eventually it should be possible to decide whether the gene is composed of several molecules (or unit particles), one of which may change at a time At present we do not know the exact nature of this alteration We do know that it can be affected by outside agencies such as x rays (30) and by the administration of toxic substances such as colchicine the nitrogen mustards, and some carcinogens

There seem to be certain normal rates of mutation for each gene These may be so low that mutations are not observed often enough to enable us to state what the normal rate of mutation is, although more extensive

observations would nearly always enable us to ascertain this There have not been many direct reports of new gene mutations (15 29) in man but there is evidence (15) from reliable pedigrees that certain human gene mutations are occurring with some frequency

Haldane (15) estimated that the normal allele of the hemophilic gene mutates to the hemophilic gene about once in thirty thousand individuals per generation Without such mutations since hemophilia is a very serious disadvantage and since the action of natural selection is to eliminate such a gene the trait would certainly have disappeared long ago Gunther and Penrose (13) estimated that the normal allele of the epiloia gene (epiloia is a rare disease in which mental defect and epilepsy are associated with tumor formation in the brain the skin and certain viscera mutates to the gene for epiloia at a somewhat lower rate

BLOOD GROUPS

The A, B, O Blood Groups

The four classical blood groups, O, A B, AB, were discovered in 1900 to 1902 by Dr Karl Landsteiner and pupils The red blood corpuscles of certain individuals are acted upon by substances present in the plasma of certain other persons in such a way to form clusters and clumps These clumps are at first so small that they can be seen only under the microscope, but when the reaction is strong they grow to a size easily discernible by the naked eye The chemical substances in the red corpuscles which permit their being agglutinated in this way are the agglutinogens Corresponding agglutinins anti A and anti B are found in plasma

The division of all persons into four blood groups depends upon the fact that the two different blood corpuscle agglutinogens, A and B, can be present singly or together, or can be absent If we designate the absence of both by O we have four possibilities O, A, B, and AB The relation of the serum agglutinins anti A and anti B to the characteristic blood corpuscle substances of the individual is given by the Landsteiner rule *There is always found that agglutinin or agglutinins which could co-exist physiologically with the blood corpuscle characteristic which is present* Thus, for example anti A is found in the presence of O and B, but not of A These relations are illustrated by table 17 in the last column of which the blood group serum characteristics are presented (see fig 12) In determining blood groups the corpuscle suspension of the person to be tested is allowed to react, preferably in the test tube (38, 16), with sera which have the property of agglutinating cells containing blood group factors A and B, respectively Typical agglutination is shown in figure 13 The group is determined by the simple scheme shown in table 18 Blood grouping tests can also be carried

TABLE 17

The human blood groups

BLOOD GROUP	AGGLUTINOGEN IN CORPUSCLES	AGGLUTININ IN SERUM
O	O	anti A and anti B
A	A	anti B
B	B	anti A
AB	AB	—

TABLE 18

Determination of groups with two test sera, anti A and anti B

	KNOWN SERUM ANTI A	KNOWN SERUM ANTI B	GROUP
Agglutination of the unknown blood corpuscles	−	−	O
	+	−	A
	−	+	B
	+	+	AB

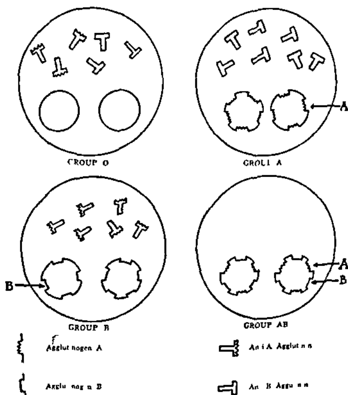


Fig 12 Schematic representation of blood agglutinogens (antigens) and agglutinins

out on glass slides instead of in test tubes. This method is often used for clinical tests in hospitals. An idea of the appearance of the reactions to the naked eye and under the microscope is given in figure 14.

Inheritance. The mechanism of inheritance was shown by Bernstein (4) to depend upon a series of three allelic genes which are designated as A, B and O. Since each person must possess some combination of two of

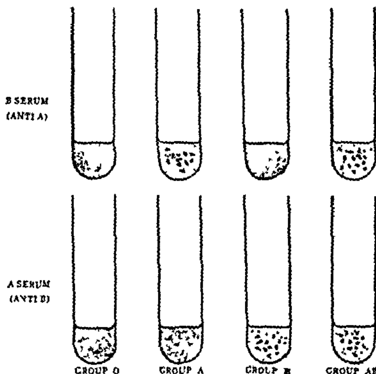


FIG. 13. Schematic representation of blood grouping as carried out in test tubes.

these genes, six different combinations—OO, AA, AO, BB, BO, and AB—are possible. Since, however, the factor O seems to be recessive to both A and B (or at least its presence in the genetic make-up of the individual does not interfere with the full expression of the A or B characteristic), the heterozygotes AO and BO are indistinguishable from the homozygotes AA or BB, respectively, and the six gene combinations produce only four distinguishable groups, as shown in table 19.

Group AB is homogeneous in the sense that all individuals in the group are alike in having one A gene and one B gene. An individual of group

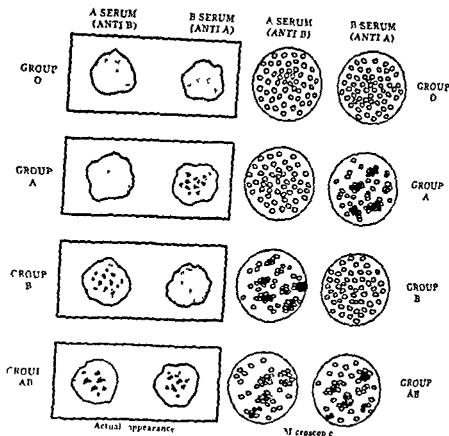


Fig 14 Schematic representation of blood grouping on slides

TABLE 19

Genotypes and phenotypes of blood group genes

GENOTYPE	BLOOD GROUP (PHENOTYPE)
OO	O
AA AO	A
BB BO	B
AB	AB

AB can therefore never become the parent of a child of group O. The blood group of an individual never changes. Table 20 shows how nearly the experimental results conform to this, the Bernstein theory.

From the absence of normal children who have a blood group incompatible with that of their mother (and the same argument applies to the M and N types discussed on page 390) we may infer that the blood group genes do not mutate at any rapid rate, for otherwise children incompatible with their mothers would have been observed. Blood groups thus may be more

TABLE 20

Inheritance of blood groups, as found in actual studies (38)

GROUPS OF PARENTS	NUMBER OF FAMILIES	NUMBER OF CHILDREN IN GROUP				
		O	A	B	AB	Total
O x O	1465	3355	(14)	(9)	0	3378
O x A	2047	2156	3389	(10)	(2)	5557
O x B	1365	1315	(7)	1690	(1)	3013
O x AB	504	(6)	607	612	(3)	1228
A x A	1270	516	2354	0	(1)	2871
A x B	1299	561	908	734	782	2985
A x AB	419	0	478	239	279	996
B x B	536	188	(1)	975	0	1164
B x AB	304	(2)	171	353	255	781
AB x AB	57	0	26	39	63	128
Totals	9806	8429	7955	4687	1378	22 427

Numbers in parentheses are apparent exceptions to the accepted theory of blood group inheritance. They are doubtless due either to faulty technic in making the tests or to illegitimacy.

"conservative" characters than albinism and hemophilia, both of which seem to be recruited steadily by mutations from the normal gene.

The genes A and B cause the production in the red blood cells of the chemical substances agglutinogens A and B, respectively. The chemical nature of these substances has been investigated by Kabat (21) and by Morgan. As isolated, they are mucopolysaccharides.

Racial Classification

Tens of thousands of individuals have been blood grouped since 1900. The very early tests did not seem to reveal very much difference in the frequencies of the four groups in various nationalities, but during the World War I two Polish doctors named Hirszfeld, working on the Macedonian battlefront, made the discovery that the percentages of the four groups varied considerably in different races. As characters for use in an

thropological classification the blood groups offer several advantages (6a) (a) they are inherited in a known way according to Mendelian principles (b) they are not altered by illness, medical treatment, of differences in climate or food (c) their frequency in a population is a very stable characteristic (d) they probably arose very early in the course of man's evolution (e) there is a considerable correlation between geography and the distribution of the blood groups and (f) the blood groups are sharply distinguishable 'all-or none characters which do not grade into each other

The only racial differences we find or can expect to find will be differences in *frequencies* of the four groups among various populations The human species is an ancient one and has presumably dispersed to its present habitats from some relatively restricted area or areas in which it originated by evolution from its ancestral anthropoid stock or stocks In the process of this dispersal there has constantly been a great deal of *mixture among different groups of men*

Other Blood Groups

M, N blood types The A B and O series of allelomorphs does not by any means exhaust the list of genes which influence the blood agglutinogens Another series discovered in 1927 is known as M and N (22) These letters represent inherited agglutinogens detectable in human blood by the use of agglutinins formed by rabbits injected with M positive (or N positive) human red cells Each human being as either two M genes, two N genes or one of each three types are thus determined as shown in table 21

Unlike the A B groups the M and N blood types as they are called have but little importance in the performance of blood transfusions since agglutinins capable of reacting specifically with these agglutinogens are rarely found in normal human blood

Subdivisions of blood groups A and AB The blood factor A is not always exactly the same in different individual human bloods There are two main varieties of A which are designated as A_1 and A_2 (38 46) Of these the latter gives weaker reactions with the average anti A reagent The variations are expressed also in the group AB so that we find two subgroups A_1B and A_2B where the difference in the A antigen is very much the same as it was in group A save that in certain instances the A reaction in individuals of subgroup A_2B may be much weaker than in any individual of group A_1 or even A_2 It has been proposed (41) that the subgroups of A are inherited in the following way (shown in table 22) Instead of the gene series A B and O suppose we have a series of four allelomorphs A_1 A_2 B and O with A_1 being dominant over A_2 and A_1 A_2 and B all being dominant over O This theory on the whole seems to fit most of the ob-

The Rh sern

and their possible outcomes being

RhRh × RhRh → 100 per cent RhRh
RhRh × Rhrh → 50 per cent RhRh, and 50 per cent Rhrh
Rhrh × Rhrh → 25 per cent RhRh, 50 per cent Rhrh and 25 per cent rhrh
RhRh × rhrh → 100 per cent Rhrh
Rhrh × rhrh → 50 per cent Rhrh and 50 per cent rhrh
rhrh × rhrh → 100 per cent rhrh

TABLE 21

The M, N blood types

GENOTYPE	PHENOTYPE
MM	M
NN	N
MN	MN

TABLE 21a

Rh genes and their reactions with anti Rh and anti Hr antisera according to Wiener's nomenclature

GENE	REACTION WITH SERUM					
	anti rh	anti Rh_0	anti rh″	anti hr′	anti Hr_0	anti hr″
r	−	−	−	+	+	+
r	+	−	−	−	+	+
r	−	−	+	+	+	−
r^y	+	−	+	−	+	−
R^o	−	+		+	−	+
R^1	+	+	−	−	−	+
R^2	−	+	+	+	−	−
R^z	+	+	+	−	−	−

However, it was soon found that the Rh factor was antigenically and genetically complex Wiener (47) now supposes that at least eight allelomorphic genes are involved, as follows

$$R^1, R^2, R^o, r', r'', r^y, R^z, r$$

The first seven are dominant over rh, but not over each other The genes R^1 and R^2 are "double acting" genes, the former producing the antigens rh′ and Rh°, the latter rh″ and Rh°

The discovery of an agglutinin which reacted regularly with Rh negative bloods led to the postulate that Rh negative bloods contained an Hr factor (Hr being the letters Rh in reverse) But this agglutinin also reacts with some of the Rh positive bloods These observations led the British

By similar tables one may work out all of the possible matings and their consequences, and a compilation of these results enables us to draw up table 24 It will be noted that some types of children can not be born to mothers of certain types For instance, a mother of blood group O can not have an AB child Consequently, we may abstract from table 24 the in

TABLE 24

Blood groups of offspring possible or impossible from any mating combination

MATING COMBINATION	ALLEGED FATHER	KNOWN MOTHER	CHILDREN POSSIBLE FROM THEIR MATING	CHILDREN NOT POSSIBLE FROM THEIR MATING DECISIVE FOR NON PATERNITY	IMPOSSIBLE FROM THIS MOTHER IN ANY MATING
1	O	O	O	A, B, (AB)	AB
2	O	A	O, A	B, AB	
3	O	B	O, B	A, AB	
4	O	AB	A, B	AB, (O)	O
5	A	O	O, A	B, (AB)	AB
6	A	A	O, A	B, AB	
7	A	B	O, A B, AB		
8	A	AB	A B AB	(O)	O
9	B	O	O B	A, (AB)	AB
10	B	A	O, A B, AB		
11	B	B	O, B	A, AB	
12	B	AB	B A AB	(O)	O
13	AB	O	A B	O, (AB)	AB
14	AB	A	A, B AB	O	
15	AB	B	A, B, AB	O	
16	AB	AB	A, B, AB	(O)	O

non paternity deducible from these blood groupings

formation which enables us to establish the non paternity of an accused man, and this is shown in table 25

In carrying out tests in such cases it is important that competent workers using known sera of known potency and specificity should do the work

The M and N types are inherited as outlined above and may be applied in exactly the same way to the exclusion of paternity in the case of an innocent man This is illustrated in table 26 Testing for the M and N factors is slightly more difficult than for the A and B groups, and not all workers are able to prepare adequate testing reagents, so these tests should certainly never be entrusted to any except experts

From the sketch of the inheritance of the Rh types it is obvious that

anti c, anti d, and anti-e are designated as anti hr′, anti Hr_o, and anti hr″ The only blood which is correctly called completely Rh negative would react with none of the three anti Rh sera, anti C, anti D, or anti E and as can be seen from tables 1 and 2, would react with all three of the anti hr sera If we consider all 6 of the known sera, then, there is no blood which does not react with at least three of them

TABLE 23

Diagrammatic examples of the hereditary transmission of the factors that determine certain blood groups

I MATING AA x AA	II AA x AO	III AO x BO	IV OO x AB
A A	A O	B O	A B
A AA AA	A AA AO	A AB AO	O AO BO
A AA AA	A AA AO	O BO OO	O AO BO
Progeny 100% group A	100% A	25% O 25% A 25% B 25% AB	50% A 50% B

On the top of each diagram are designated the factors possessed (and transmissi … parent does not matter … he letters … of a child unless it was present in the blood of at least one of its parents (4) Diagrams of all the 21 possible types of mating have been tabulated (38, 46)

Application of Blood Groups in Cases of Disputed Parentage

Knowing the rules of inheritance of the blood groups it is possible to establish in some cases that a man falsely accused of being the father of an illegitimate child is in reality innocent It is obvious that we can not expect to prove by blood groups that he is the father, since it is always possible that some other man having the same blood group is the father

The rules of heredity of blood groups may be applied to problems of disputed paternity First of all we may give four examples of possible types of mating and the types of children which would result from them All that is necessary is to know that each parent contributes one gene of a pair to the offspring and that the factors A and B may be considered as dominant over O (table 23)

itself many times in the course of the development of the organism to maturity. Therefore, one of the most significant synthetic activities during growth is the duplication of genes, each gene making an exact copy of itself, selecting and utilizing just those food materials which are necessary to reproduce its own specific properties.

The most concrete theory of self-duplication of genes applicable also to the formation of proteins, enzymes, and antibodies is that of Haurowitz (17). He has stated that the first step of protein synthesis is essentially the formation of a positive replica of a primary template both present as

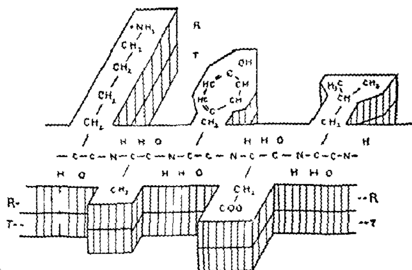


FIG. 15. Second step of protein synthesis according to Haurowitz.

expanded monomolecular films and the second step is the formation of a negative globular replica complementarily adapted to a secondary globular template.

An essential part of the view of Haurowitz is that the primary template consists of an expanded protein layer. The mechanism which maintains the template protein film in the expanded state is believed to involve the nucleic acids, which are always found in quantity at the sites of protein synthesis. These nucleic acids cause the peptide chains to unfold and to remain in the expanded, unfolded state of the monomolecular micelle.

The second step of protein synthesis, and thus would apply to the duplication of a gene, is shown in figure 15. Each of the amino acids of the template layer attracts another amino acid of the same type from the surrounding medium, and the amino acids forming this second layer are

disputed parentage may be tested by determination of these types also However, the number of possible matings is very large and a table corresponding to those given would occupy far too much space Furthermore relatively few laboratories in the country are equipped to do these delicate tests therefore it seems best to be content with a statement that the tests and their interpretation should be made only by experts

Several states have passed laws making it possible for the court to order

TABLE 25

Combinations allowing the man to establish non paternity omitting instances of impossible mother child combinations (condensed from table 24)

PUTATIVE FATHER	KNOWN MOTHER	KNOWN CHILD
O	O	A B
O	A	B AB
O	B	A AB
O	AB	AB
A	O	B
A	A	B AB
B	O	A
B	B	A AB
AB	O	O
AB	A	O
AB	B	O

TABLE 26

Exclusion of paternity on the basis of characteristics M and N

CHILD	MOTHER	FATHER NOT POSSIBLE
M	M or MN	N
N	N or MN	M
MN	M	M
MN	N	N

the participants of a suit involving paternity to submit to blood grouping tests and directing that these tests may be received as evidence if they exclude paternity They of course can not be construed as proving paternity Other states which do not have such laws usually admit the evidence when it is offered

THE SELF-DUPLICATION OF GENES

An organism ordinarily arises from a zygote which has two representatives at each gene locus In the course of its development each cell gives a replica of this original set of genes which therefore can be seen to reproduce

6 Boyd, W C Assay of blood grouping sera Variations in reactivity of cells of different individuals belonging to groups A and B Ann New York Acad Sc, 46: 927-937, 1946
6a Boyd, W C *Genetics and the Races of Man* Boston, Mass, Little, Brown & Co, 1950
7 Boyd, W C, and Boyd, L G Sexual and racial variations in ability to taste phenyl thiocarbamide with some data on the inheritance Ann Eugenics, 8 46-51, 1937
7a Chang, M C, and Pincus, G Physiology of fertilization in mammals Physiol Rev, 31: 1-26, 1951
8 Chetverikov, S S On certain features of the evolutionary process from the viewpoint of modern genetics J Exper Biol (Russian), 2. 3 54, 1926
9 Falconer, D S Sensory thresholds for solutions of phenyl thiocarbamide Results of tests on a large sample made by R A Fisher Ann Eugenics, 13: 211-222, 1947
10 Fisher, R A *Genetics and the Theory of Nat ı al Selection* Oxford Eng, Clarendon Press, 1930
11 Fisher, R A Crest and hernia in fowls due to a single gene without dominance Science, 80: 288-289 1934
12 *Greenberg, B E, and Gargill, S I The relation of hyaluronidase in the* seminal fluid to fertility Human Fertil 11 1 1946
13 Gunther, M, and Penrose, L S The genetics of epiloia J Genetics 31. 413-430, 1935
14 Haldane, J B S *New Paths in Genetics* New York and London, Harper & Brothers, 1942
15 Haldane, J B S The mutation rate of the gene for hemophilia and its segregation ratios in males and females. Ann Eugenics, 13: 262 271, 1947
16 Harris, H The inheritance of premature baldness in men Ann Eugenics, 13. 172 181, 1946
17 Haurowitz, F *Chemistry and Biology of the Proteins* New York, N Y, Academic Press, 1950
18 Hudson, P B, and Butler, W W S A study of the enzyme acid phosphatase and its possible role in intermediary carbohydrate metabolism of the prostate gland and its secretions in dog and man J Urol, 63: 323-333, 1950
19 Huggins, C The prostatic secretion (review) Harvey Lect, 42. 148-193, 1946-1947
19a Huggins, C The physiology of the prostate gland Physiol Rev, 25. 281, 1945
20 Huxley, J L *Evolution, the Modern Synthesis* New York and London, Harper & Brothers, 1942
21 Kabat, E A, *et al* Immunochemical studies on blood groups IV J Exper Med, 85. 685-699, 1947
22 Landsteiner K, and Levine, P On individual differences in human blood J Exper Med, 47: 757-775, 1928
23 Landsteiner K, and Wiener, A S An agglutinable factor in human blood recognized by immune sera for rhesus blood Proc Soc Exper Biol & Med 43: 223 224, 1940
24 Levine, P, and Katzin, E M Isoimmunization in pregnancy and the varieties of isoagglutinins Proc Soc Exper Biol. & Med, 45. 343 346 1940
25 Lillie, F. R *Problems of Fertilization* Chicago, Ill, University of Chicago Press, 1919

linked together by the action of enzyme mixtures of the cathepsin type The decisive step in the whole mechanism is the first phase of this second stage of the process—that is, the specific adsorption of amino acids to amino acids of the same type This is very analogous to the phenomena which are observed in crystallization, and we know that crystallization is extremely specific

Haurowitz points out that the discovery of Cori and Cori of the enzymatic synthesis of polysaccharides from glucose 1 phosphate supports his concept It was found by Cori and Cori that the enzymatic synthesis of polysaccharides depends upon what type of primer was added If a small *amount of some branched chain carbohydrate is added as primer, branched* polysaccharides will be formed If a straight chain polysaccharide is added straight chain polysaccharides are formed The added material apparently acts as seed for the material to be formed, and is thus called a starter or primer Thus the specificity of the formation of proteins and genes lies not only with the catalyst involved but with the organizers or primers These consist of non catalytic molecules which act merely as the elementary units of a crystal lattice and direct, so to speak, the apposition of other building stones

The third stage of Haurowitz' hypothetical formation of proteins and genes involves the casting off of the two dimensional replica which then is free to fold up into a three dimensional globular protein molecule The template is then available for the production of other replicas

Haurowitz points out that if the genes are identical with the primary two-dimensional templates of protein synthesis, we must assume that the first phase of protein synthesis the formation of the two-dimensional replica goes on in the nucleus, for genes are present only in the nuclei We do not know where the second phase of protein synthesis, formation of the globular three dimensional molecule from the two-dimensional replica, takes place

REFERENCES

1 BATESON W AND PUNNETT, R C On the interrelations of genetic factors Proc Roy Soc London, s B 84 3-8 1911

Chem Rev 37 15-96 1945

for colour blind

123 119 1937

erblichen Blut

bungslehre 37

227-270 1925

4a of des

eredity

114 12

5 BOYD W C Rh blood factors

1945

PART IV

Metabolism

26 MALKIEL, S, AND BOYD, W C A transfusion reaction due to a dangerous universal donor J A M A 129 [illegible]

27 MAN [illegible]
of [illegible]

28 MENDEL, G J *Experiments in Plant Hybridization* (Trans by Royal Horticultural Society, London) Harvard University Press, Boston, Mass 1918

29 [illegible] 931

30 [illegible]

31 [illegible] 897 921, 1929

32 MULLER, H J Types of visible variations induced by x rays in Drosophila J Genetics, 22: 299-334, 1930

33 PEARL, R Biology and social trends J Wash Acad Sc, 25 No 6 253-296, 1935

34 RACE, R R, AND SANGER, R *Blood Groups in Man* Oxford, Eng, Blackwell 1950

35 RIFE, D C A common misconception concerning gamma behavior of linked factors Human Biol 11. 546-548 1939

36 RUNDALES, R W, AND FALLS, H F Hereditary (? sex linked) anemia Am J M Sc, 211: 641 658 1946

37 RUSSELL, E S *The Interpretation of Development and Heredity* Oxford, Eng, Clarendon Press 1930

38 SCHIFF, F, AND BOYD W C *Blood Grouping Technic* New York, N Y, Interscience Publishers 1942

38a SINNOTT, E W et al *Principles of Heredity* New York, N Y, McGraw Hill, 1950

39 STRANDSKOV, H H Human genetics and anthropology Science, 100 570-571 1944

40 TAYLOR, G L, AND RACE, R R Human blood groups Brit M Bull, 2 160-164, 1944

41 THOMSEN, O, et al Über die möglichkeit der Existenz zweier neuer Blutgruppen, auch ein Beitrag zur Beleuchtung sogenannter Untergruppen Acta path et microbiol scandinav 7. 157-190, 1930

42 VON TSCHERMAK, E The rediscovery of Gregor Mendel's work J Hered, 42 163-171, 1951

43 TYLER, A Properties of fertilizin and related substances of eggs and sperm of marine animals Am Naturalist, 83 195-219, 1949

44 WADDINGTON, C H *An Introduction to Modern Genetics* New York, N Y, Macmillan Co 1939

45 WALSH, R J AND MONTGOMERY, C M A new human iso agglutinin subdividing the MN blood groups Nature 160. 504, 1947

46 WIENER, A S *Blood Groups and Transfusion* 3rd edit Springfield, Ill, Charles C Thomas, 1943

47 [illegible]

47a [illegible] of blood of homologous group, with three cases in which same agglutinogen was responsible Ann Int Med, 13. 2306-2322, 1940

48 WRIGHT, S Physiological and evolutionary theories of dominance Am Naturalist, 68. 24 53, 1934

49 WRIGHT, S The physiology of the gene Physiol Rev, 21: 487-527, 1941

CHAPTER 10

Food and Diet

The various foods of man, excepting water and purely mineral items, are the anabolized products of other living organisms. In this respect man, like all animals, is a saprophyte. The various animal products in man's diet, whether it be whale blubber, ants, honey, milk, lobsters, or pork chops, are derived from organisms that, in turn, must produce their tissues from raw materials in the form of the green plant or from still other animals that feed on the green plant. No matter how long the chain of animals that eat and are eaten, the primary source of food can be traced, eventually, to the green plant.

Whence does the green plant obtain its fat, protein, and carbohydrate? Upon what does it feed? The answer is that its own raw materials, or food, consist of carbon dioxide from the air, and the water and water-soluble mineral content of the soil. From these relatively simple materials it constructs the complex substances discussed in the first part of this text. The synthesis of protein from carbon dioxide, water, nitrates, and sulfates, to take the most startling example, is an anabolic process which requires the input of considerable energy. This is certain, since the oxidation of proteins back to carbon dioxide, water, and inorganic material liberates considerable energy. Where does the plant obtain this energy? The answer is that plant anabolism is made possible through the absorption and utilization of the energy of sunlight. The process which utilizes and stores this energy is known as *photosynthesis*.

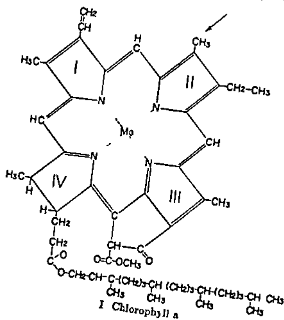
PHOTOSYNTHESIS

Chlorophyll

Photosynthesis in green plants is dependent upon the presence of *chlorophyll*, a green pigment. There is significance, therefore, in the adjective "green" when we speak of plants in connection with photosynthesis, since plants which do not have chlorophyll, for instance, the various mushrooms and yeasts, cannot photosynthesize. Accompanying chlorophyll are various carotenoids, the yellow or red color of which is masked by the chlorophyll green. The chlorophyll content of most leaves is about one per cent of the

Despite these differences the similarities between the two substances are far more striking and it is interesting to note the economical manner in which evolutionary forces adapt a single molecular framework to such vastly different yet equally important chemical roles in plants and animals.

Chlorophyll is not a chemical individual. The one carbon substituent on pyrrol nucleus II may be a methyl group as in heme, in which case we have



I Chlorophyll a

Note. Replacement of the $-CH_3$ group (indicated by the arrow) with a $-C\genfrac{}{}{0pt}{}{\diagup O}{\diagdown H}$ group will result in a chlorophyll b molecule.

chlorophyll a, or it may be an aldehyde group, in which case we have *chlorophyll b*. Chlorophyll *a* is slightly more soluble in fat solvents than chlorophyll *b*. Their solubility pattern differs from that of the simple lipids in that they are fairly soluble in 95 per cent alcohol, and only difficultly soluble in petroleum ether. The chlorophylls *a* and *b* vary also in their absorption spectra. Chlorophyll *a* absorbs almost twice as strongly in the red, for instance. The ratio of the *a* and *b* varieties in most higher plants is about three to one. In algae there are species variations and in addition, other closely related varieties of chlorophyll.

dry weight, while the carotenoids are present in concentrations as little as one eighth to as much as one half that of chlorophyll

Chlorophyll in its native state within the *chloroplast* (that plant cell organelle which contains the pigment) is probably in close association with protein, lipid, or both The catalyst of the photosynthetic process may thus be viewed as a compound lipoprotein of which chlorophyll and various carotenoids are the prosthetic groups or as an enzyme of which these are the coenzymes

Nor is it an accident that the prosthetic groups are colored Since plant anabolism uses light as the source of energy, some mechanism must exist for the absorption of light The structure of chlorophyll is such that it absorbs light in the purple and red, the non absorbed portion of the spec trum being reflected for the most part and appearing green to our eyes The function of the carotenoids may well be to absorb some of the light rejected by the chlorophyll The pigments would thus behave, primarily, as "energy traps", while the lipoprotein moiety would be the photosynthetic enzyme proper

Despite all efforts the enzyme system, as an undenatured whole, has not yet been isolated from the chloroplast Chlorophyll by itself, although comparatively easy to isolate in quantity, is powerless to effect the photo synthetic transformation, so that photosynthesis has not yet been dupli cated in the test tube and visions of a future in which man s ultimate food supply can depend on his chemical industries alone are as yet remote

The chemical structure of chlorophyll (formula I) is remarkably similar to that of the heme of hemoglobin (see Chapter 4) It is built upon the porphyrin nucleus but differs from heme in the following respects

(1) The carboxyl group of pyrrol nucleus IV is esterified with the long chain alcohol *phytol* Phytol ($C_{20}H_{39}OH$) is built up of isoprene units and is, therefore, related to the carotenoids, a fact which may possibly be *significant in view of the close association of* carotenoids and chloro phyll within the leaf The porphyrin and phytol portions of the molecule are respectively polar and non polar, so that the former can associate with the protein and the latter with the lipid in the photosynthesizing lipoprotein Pyrrol nucleus IV also contains two more hydrogen atoms than does the analogous ring in heme

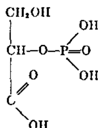
(2) The propionic acid group of pyrrol nucleus III is oxidized and condensed with a —CH= group to form a five carbon ketone ring It is further esterified with methyl alcohol

(3) The two carbon substituent on pyrrol nucleus II is an ethyl group rather than a vinyl group as in heme

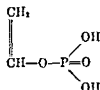
(4) The metal associated with the four nitrogens of the pyrrols is magnesium, and not iron as in the case of the hemes.

hydrate produced was by far the more difficult problem and before the days of radioactive tracers guesses ranged from such simple products as formaldehyde to such complex ones as starch Monosaccharides, disaccharides and polysaccharides all exist in the leaf and such is the speed of photosynthesis that it was difficult to show that any of them were formed before any of the others

Calvin (2) using radioactive carbon 14 as the raw material, marine algae as the photosynthesizing cell, and paper chromatography as the means of separation of products, attempted to identify the primary products by allowing photosynthesis to proceed for very short periods after supplying the radioactive carbon dioxide before killing and analyzing the plants It is interesting to note that in a minute and a half 90 per cent of the carbon dioxide had been reduced and no less than fifteen different radioactive organic products could be isolated In five seconds of photosynthesis five



III 2 Phosphoglyceric acid



IV Vinyl phosphate

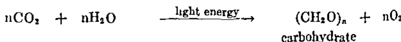
compounds could be detected malic acid aspartic acid pyruvic acid, 3 phosphoglyceric acid and 2 phosphoglyceric acid Sixty five per cent of the radioactive carbon occurred in the last two compounds If the temperature is lowered and photosynthesis is allowed to proceed at 4°C, only one compound can be isolated and that one is 2 phosphoglyceric acid (formula III) While this does not constitute proof that it is the very first organic compound formed in photosynthesis (indeed, vinyl phosphate (formula IV) may be its precursor), it is certainly the earliest that has yet been located

Calvin has presented a scheme of anabolism, a simplified version of which is presented in formula IV a Some of the steps, Calvin admits, are as yet completely hypothetical Note that the scheme is essentially that of a cycle (steps 1 to 11 in the formula) beginning and ending with vinyl phosphate During the cycle, however two molecules of CO_2 are added (steps 1 and 3) to form a four-carbon chain This four-carbon chain is broken in reaction 6 to two two-carbon molecules The net result is that at each turn of the cycle an additional molecule of vinyl phosphate is produced If all the

The Photosynthetic Reaction

The fundamental photosynthetic reaction (formula II) involves the reduction of carbon dioxide The production of carbohydrate (which is the measure of successful photosynthesis) requires carbon dioxide water, and energy quanta at least as energetic as those of red light Note that a molecule of oxygen is produced for each molecule of carbon dioxide consumed Experiments with isotopic oxygen have shown that the liberated oxygen is derived from the water molecule, not from the carbon dioxide

Photosynthesis thus not only serves to prepare the food supply for all animal life, but, in addition, renews the oxygen supply of the atmosphere as it is consumed by the catabolic processes of life It has been estimated that photosynthetic processes are sufficient to renew all the oxygen in the air in a little over two thousand years and to decompose all the water in the oceans in about two million years (15) It is also interesting to note that the contribution of marine algae to the utilization of atmospheric carbon dioxide is eight times that of the land vegetation we usually think of in connection with photosynthesis (15)



$$nCO_2 + nH_2O \xrightarrow{\text{light energy}} \underset{\text{carbohydrate}}{(CH_2O)_n} + nO_2$$

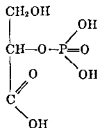
II Photosynthesis—over all reaction

The nature of the "reduced carbon" produced in photosynthesis is by no means an easy problem to solve for two reasons at least In the first place, the intact cell must be used in photosynthesis experiments and simple components of the system can not be isolated for leisurely study Secondly, the reactions that take place during photosynthesis are amazingly rapid Using radioactive carbon dioxide, one can detect radioactivity in fats, carbohydrates, and proteins in the leaf within two minutes In fact, were it not for the use of radioactive tracers and for the technique of paper chromatography in separating the complex mixture of photosynthetic products, solution of the problem would be all but hopeless

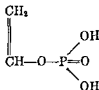
Efforts have been numerous and strenuous to determine the nature of the "primary photochemical process", that is, the first reaction involving the photochemical reduction of carbon dioxide and the nature of the organic product formed From the fact that one molecule of oxygen is formed for every molecule of carbon dioxide consumed, and that the empirical formula for the organic residue must therefore be CH_2O, it was early concluded that photosynthesis must result in the production of carbohydrate, and that lipids and proteins must be produced from carbohydrates by non photosynthetic mechanisms The nature of the first carbo-

hydrate produced was by far the more difficult problem and before the days of radioactive tracers guesses ranged from such simple products as formaldehyde to such complex ones as starch Monosaccharides, disaccharides and polysaccharides all exist in the leaf and such is the speed of photosynthesis that it was difficult to show that any of them were formed before any of the others

Calvin (2), using radioactive carbon 14 as the raw material, marine algae as the photosynthesizing cell, and paper chromatography as the means of separation of products, attempted to identify the primary products by allowing photosynthesis to proceed for very short periods after supplying *the radioactive carbon dioxide before killing and analyzing the plants* It is interesting to note that in a minute and a half 90 per cent of the carbon dioxide had been reduced and no less than fifteen different radioactive organic products could be isolated In five seconds of photosynthesis five



III 2 Phosphoglyceric acid



IV Vinyl phosphate

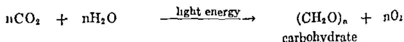
compounds could be detected—malic acid, aspartic acid, pyruvic acid, 3 phosphoglyceric acid, and 2 phosphoglyceric acid Sixty five per cent of the radioactive carbon occurred in the last two compounds If the temperature is lowered and photosynthesis is allowed to proceed at 4°C, only one compound can be isolated and that one is 2 phosphoglyceric acid (formula III) While this does not constitute proof that it is the very first organic compound formed in photosynthesis (indeed, vinyl phosphate (formula IV) may be its precursor), it is certainly the earliest that has yet been located

Calvin has presented a scheme of anabolism, a simplified version of which is presented in formula IV a Some of the steps, Calvin admits, are as yet completely hypothetical Note that the scheme is essentially that of a cycle (steps 1 to 11 in the formula), beginning and ending with vinyl phosphate During the cycle, however, two molecules of CO_2 are added (steps 1 and 3) to form a four carbon chain This four carbon chain is broken in reaction 6 to two two carbon molecules The net result is that at each turn of the cycle an additional molecule of vinyl phosphate is produced If all the

The Photosynthetic Reaction

The fundamental photosynthetic reaction (formula II) involves the reduction of carbon dioxide The production of carbohydrate (which is the measure of successful photosynthesis) requires carbon dioxide water, and energy quanta at least as energetic as those of red light Note that a molecule of oxygen is produced for each molecule of carbon dioxide consumed Experiments with isotopic oxygen have shown that the liberated oxygen is derived from the water molecule, not from the carbon dioxide

Photosynthesis thus not only serves to prepare the food supply for all animal life, but, in addition, renews the oxygen supply of the atmosphere as it is consumed by the catabolic processes of life It has been estimated that photosynthetic processes are sufficient to renew all the oxygen in the air in a little over two thousand years and to decompose all the water in the oceans in about two million years (15) It is also interesting to note *that the contribution of marine algae to the utilization of atmospheric* carbon dioxide is eight times that of the land vegetation we usually think of in connection with photosynthesis (15)



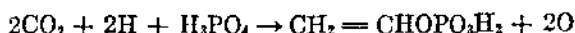
$$nCO_2 + nH_2O \xrightarrow{\text{light energy}} \underset{\text{carbohydrate}}{(CH_2O)_n} + nO_2$$

II Photosynthesis—over all reaction

The nature of the "reduced carbon" produced in photosynthesis is by no means an easy problem to solve for two reasons at least In the first place, the intact cell must be used in photosynthesis experiments and simple components of the system can not be isolated for leisurely study Secondly, the reactions that take place during photosynthesis are amazingly rapid Using radioactive carbon dioxide, one can detect radioactivity in fats, carbohydrates, and proteins in the leaf within two minutes In fact, were it not for the use of radioactive tracers and for the technique of paper chromatography in separating the complex mixture of photosynthetic products, solution of the problem would be all but hopeless

Efforts have been numerous and strenuous to determine the nature of the "primary photochemical process", that is, the first reaction involving the photochemical reduction of carbon dioxide and the nature of the organic product formed From the fact that one molecule of oxygen is formed for every molecule of carbon dioxide consumed, and that the empirical formula for the organic residue must therefore be CH_2O, it was early concluded that photosynthesis must result in the production of carbohydrate, and that lipids and proteins must be produced from carbohydrates by non photosynthetic mechanisms The nature of the first carbo-

steps are summed up (remembering that after step 6 two of each indicated molecule exist for every original vinyl phosphate), the over all equation can be written as follows



$$2CO_2 + 2H + H_3PO_4 \rightarrow CH_2 = CHOPO_3H_2 + 2O$$

Essentially, then, the scheme represents the reduction of CO_2 and the conversion in the process of inorganic phosphate into the high-energy phosphate bond of vinyl phosphate (an enol phosphate, see Chapter 3) Light energy is thus converted into chemical energy Calvin is not yet prepared to say at which stage of the cycle the primary photochemical energy contribution takes place

Note further that there are several exits from the cycle Chemical energy can be stored in quantity as carbohydrates through 3 phosphoglyceric acid, as lipids through acetyl phosphate or converted to other forms of energy through the Krebs cycle The addition of ammonia can form such amino acids as glycine alanine and serine (as well as others in reactions not shown in the diagram)

The efficiency of photosynthesis, taking into consideration the quantity of light energy actually absorbed by the green leaf, is remarkably high according to the investigations by Warburg and Burk (20) The investigators found that 4 quanta of red light were absorbed for each molecule of carbon dioxide hydrated, which is equivalent to saying that 65 per cent of the light energy is stored as chemical energy Such efficiencies are markedly higher than those of photochemical reactions known to laboratory chemists The one most closely approaching photosynthesis is the photochemical oxidation of oxygen to ozone which shows an efficiency of 50 per cent This last reaction, however, requires the comparatively energetic photons of ultraviolet light The fact that the chlorophyll lipoprotein of plants attains much higher efficiencies with the mild energies of red light is another indication of the delicate and adaptable tools which life has developed in the form of the protein molecule

It remains true however that such high efficiencies result from careful control of laboratory conditions—i e , the use of red light, which is well absorbed by the chloroplast in quantities just sufficient to induce optimum reaction If the efficiency is calculated taking into account absorption of sunlight which usually irradiates the leaf to excess and contains wave lengths between the red and violet which are but little utilized in the plant, the efficiency of the process is much lower Rabinowitsch estimates that the efficiency in direct sunlight is of the order of 3 per cent where absorbed light only is considered or 2 per cent when the total incident light (only two thirds of which is absorbed) is taken into consideration

$CH_2(OH)CHOH(O\text{Ⓟ}) \xrightarrow[(9)]{-H_2O} CH_3COO\text{Ⓟ} \xrightarrow{\text{(condensaton)}}$ Lipids

Glycolaldehyde Phosphate (hydrated) — Acetyl Phosphate

(8) $+ 2H$, $+$ Ⓟ: $CH_2(OH)COOH$ (Glycolic Acid) → Glycolaldehyde Phosphate (hydrated)

(10) $+ 2H$: Acetyl Phosphate → $CH_3CHOH(O\text{Ⓟ})$ (Acetaldehyde Phosphate (hydrated))

$CH_3CHOH(O\text{Ⓟ}) \xrightarrow[(11)]{-H_2O} CH_2{=}CHO\text{Ⓟ}$ (**Vinyl Phosphate**) $\xrightarrow[+H_2O\ (1)]{+CO_2} CH_2(OH)CH(O\text{Ⓟ})COOH$ (2 Phosphoglyceric Acid)

2 Phosphoglyceric Acid $+ NH_3$ → $CH_2(OH)CH(NH_2)COOH$ (Serine)

2 Phosphoglyceric Acid → $CH_2(O\text{Ⓟ})CH(OH)COOH$ (3 Phosphoglyceric Acid) (isomerization)

3 Phosphoglyceric Acid (condensation) → Carbohydrates

(2) $- H_2O$: 2 Phosphoglyceric Acid → $CH_2{=}C(O\text{Ⓟ})COOH$ (Phosphoenolpyruvic Acid)

$CH_3CH(NH_2)COOH$ (Alanine) $\xleftarrow[+2H]{+NH_3}$ $CH_2{=}C(O\text{Ⓟ})COOH$ (Phosphoenolpyruvic Acid)

(7) $+ 2H$: $CHOCOOH$ (Glyoxylic Acid) → $CH_2(OH)COOH$ (Glycolic Acid)

$CHOCOOH \xrightarrow[+ 2H]{+ NH_3} CH_2(NH_2)COOH$ (Glycine)

(3) $+ CO_2$, $+ H_2O$: Phosphoenolpyruvic Acid → $HOOCCH_2COCOOH$ (Oxaloacetic Acid) → Krebs Cycle (see Chapter 12)

(4) $HOOCCH(OH)CH(OH)COOH$ (Tartaric Acid) $\xleftarrow{+H_2O}$ $HOOCCH_2COCOOH$

(5) $HOOCC(OH){=}C(OH)COOH$ (Dioxymaleic Acid) $\xleftarrow{-2H}$ $HOOCCH(OH)CH(OH)COOH$

(6) (split in half): Dioxymaleic Acid → Glyoxylic Acid

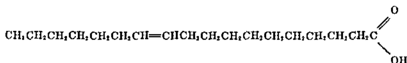
Note Ⓟ symbolizes the phosphate radical, $-PO_3H_2$

A Suggested Scheme of Anabolism in the Green Plant Cell, after Calvin (2)

Formula IV a

diet which is deficient in the naturally occurring fatty acids more unsaturated than oleic acid Addition to this diet of linoleic acid removes the symptoms Hansen and Burr (3) summarized the current state of knowledge of these unsaturated fatty acid deficiencies in humans The results on human beings were in no way conclusive due to the small number of subjects and the general difficulty of conducting dietary experiments on such a poor laboratory animal as *Homo sapiens* However deficiency seemed to interfere with the maintenance of skin integrity and resulted in such manifestations as prickly heat and eczema

Hansen and Burr list linoleic acid linoleyl alcohol linolenic acid arachidonic acid docosahexenoic acid and hexahydroxystearic acid as relieving the dermatitis in small laboratory animals Of these linoleic acid linolenic acid and arachidonic acid are normal components of the diet Together they are referred to often as *essential fatty acids* The word essential is not to be given undue physiological significance The fact that oleic acid is not an essential fatty acid does *not* mean that the body can do without



V Vaccenic acid

it as part of its structure but means merely that it is not needed as such in the diet because the body can synthesize it from other fatty acids or even from glucose Confusions which frequently arise among medical students over this use of the word essential indicate how preferable it would be to term linoleic acid and related fatty acids as *fatty acid food factors* despite the overwhelming use of essential' in medical literature A similar opportunity for confusion will arise in our discussion of amino acid food factors

An unusual fatty acid *vaccenic acid* (formula V), differing from oleic acid in that the double bond is not centrally situated occurring in small quantities (less than 0 85 per cent) in butter fat, lard and beef fat may be a food factor for the rat but no data have yet been obtained on humans (1 13)

Proteins

"Complete" and "incomplete" proteins Whereas starches and lipids from various species of plants and animals differ only to a minor degree in their nutritional value, proteins are unique in that certain of them are incapable of supporting growth when they are the only protein con

FOOD FACTORS

Animal species, while capable of extensive biosyntheses, are by no means as versatile as the green plant In other words the food factors required by animals are more numerous and generally more complex than those needed by plants *Food factors* may be defined as the simplest chemical substances—i e, those of the lowest molecular weight—which will adequately nourish an organism Those food factors which are required by the body in comparatively small quantities, such as the vitamins and some, but not all, minerals may be differentiated from the rest as *micronutrients*

Carbohydrates and Lipids

Glucose. In any consideration of food factors, carbohydrates and lipids may be considered together since their metabolism follows in part, a common pathway They occur in foods in forms usually too complex for direct use, as starch or disaccharides on the one hand or as glycerides on the other In the case of carbohydrates, digestive processes break down ingested starch and disaccharides to the monosaccharides glucose, fructose and galactose, which are all readily absorbed from the intestine and are interconvertible within the body Glucose and fructose are used in parenteral or intravenous feeding Either can supply all the body's needs for carbohydrate In a practical sense, therefore, these monosaccharides may be considered the body's carbohydrate food factors, and since the sugar in blood is characteristically glucose, it can be chosen as the carbohydrate food factor *par excellence*

We may go further than this Glucose can be used in the body as the raw material for the biosynthesis of both glycerol and most of the fatty acids, *so that glucose is the lipid food factor as well* This can be demonstrated on a large scale by the fact that ingestion of large quantities of carbohydrates will result in the formation of large quantities of fat In the case of livestock this is a useful and desirable process, in the case of human beings it is somewhat less so The interconversion of carbohydrates and fat is made possible by the fact that in the catabolism of these substances a "two carbon fragment" is reached through a series of reversible reactions with

carbohydrate lipid food factor

Fatty acid food factors. Not all the fatty acids can be synthesized in the body from carbohydrates or from other fatty acids Apparently some ... bonds

ite

glycine alanine, and glutamic acid, despite their nutritional label, are integral and necessary parts of proteins—as vital to life as any of the others Their "non-essentiality" consists only of this that they can be synthesized by the body from other customary components of the diet The "essential" amino acids can not so be synthesized and must be found as such in the diet It is preferable to think of the ten amino acids listed as the amino acid food factors for the growing rat and to avoid the confusing word "essential" altogether The adult rat, where growth has ceased, no longer requires arginine in the diet The rat apparently can synthesize arginine at a rate fast enough to balance the normal rate of destruction of that amino acid but not fast enough to form additional arginine-containing protein as would be required in a growing organism

As a by product of these researches into amino acid nutrition, threonine was discovered (12) When mixtures of all amino acids known at that time (in 1935) failed to permit growth in rats, the existence of another amino acid food factor was suspected, and investigations followed which revealed the existence of threonine It was early realized that the list of amino acid food factors varied with the species of animal investigated Thus the chick in addition to the food factors required by the young rat, must be supplied dietary glycine as well Human needs in this direction can not therefore be determined by animal experimentation alone Investigations into the nature of man s amino acid food factors were begun as early as 1941 (5) More recently Rose (16) announced a complete list of such food factors for man He fed healthy graduate students on a diet consisting of maize starch, sucrose butterfat, maize oil inorganic salts, vitamins, and purified amino acid mixtures The adequacy of the diet to supply the essential needs for protein synthesis was judged by its ability to maintain nitrogen equilibrium When the loss of nitrogen through excretion was greater than that ingested it meant that the particular combination of amino acids in the artificial diet could not entirely compensate for all the amino acids being lost in normal protein catabolism If the situation could not be corrected by simple increase in the quantity of the amino acid mixture being ingested, it meant that one or more amino acid food factors were missing

By such methods it was found that the list of amino acid food factors for adult non growing humans is (1) valine, (2) leucine (3) isoleucine (4) threonine (5) methionine, (6) lysine, (7) phenylalanine and (8) tryptophane Note that compared with the list of amino acid food factors for the growing rat, both arginine and histidine are missing The absence of histidine from the list of food factors is most unexpected since it is required by all other animal species thus far studied and since the human body is not thought capable of synthesizing the imidazole ring

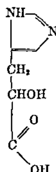
stituent of a diet otherwise adequate. Thus, rats which were fed upon a diet containing casein as the only source of protein thrived and grew at a normal rate. When gliadin, the prolamine of wheat, was substituted for casein they grew only slightly, and when zein, the prolamine of maize, was the protein source, the animals actually declined in weight.

The reason for the nutritional deficiency of some proteins is absence of, or near absence of, certain amino acids in their chemical make up. Gliadin possesses a lysine content of one per cent as compared with 6 per cent in casein, and moreover only a little over one per cent of tryptophane as compared with twice that quantity in casein. Zein lacks lysine and tryptophane entirely. From this it would appear likely that the rat can not synthesize lysine or tryptophane from other food substances but must find these ready made in the diet. That not all amino acids are of similar importance in the diet is indicated by the fact that casein is quite deficient in glycine, serine, and cystine, possessing 0.5 per cent or less of each, yet remains a protein of excellent nutritional properties. When tryptophane and lysine are added to zein, the "enriched" protein allows growth.

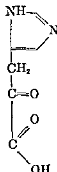
In general, complete proteins, i.e., those which are capable of supporting growth when they are the sole contributors to the protein of the diet, are from animal sources. Examples are casein and lactoglobulin from milk, ovalbumin and ovovitellin from egg, and the various proteins of meat, fish, and poultry. In addition, glycinin of soybean, excelsin of Brazil nut, glutenin of wheat, and glutelin of corn qualify. Incomplete proteins, those incapable of supporting growth, are the prolamines of wheat, barley, and rye. Zein and gelatin are examples of particularly incomplete proteins. Naturally, the classification of a protein as complete or incomplete depends upon its quantity in the diet. It must be present in sufficient amounts so that even the necessary amino acid in which it is poorest is adequate. Thus edestin, to be "complete" when fed to rats, must be fed in quantities 25 per cent greater than the amount which would be adequate for casein. If the quantity of edestin is reduced to that of casein it becomes partially incomplete.

Amino acid food factors ("essential amino acids"). The use of mixtures of purified amino acids to replace protein in experimental diets has borne out the concept of amino acids as food factors. Ten of the amino acids are indispensable in the diet of the young growing rat. These are (1) tryptophane, (2) lysine, (3) methionine, (4) threonine, (5) phenylalanine, (6) leucine, (7) isoleucine, (8) valine, (9) histidine, and (10) arginine.

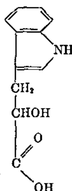
Given adequate quantities of each of these ten, the growing rat could biosynthesize all the other amino acids needed for protein production. It can not be overemphasized that "non-essential" amino acids such as



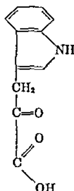
VI Imidazole lactic acid



VII Imidazole pyruvic acid



VIII Indole lactic acid



IX Indole pyruvic acid

TABLE 27

Nutritional requirements of essential amino acids

AMINO ACID	MINIMUM DAILY REQUIREMENT
	gms
L Tryptophane	0 25
L Phenylalanine	1 10
L Lysine	0 80
L Threonine	0 50
L Valine	0 80
L Methionine	1 10
L Leucine	1 10
L Isoleucine	0 70

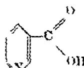
It should be kept in mind, however, that experiments such as these are of necessity relatively short term affairs There is no certainty that the apparent absence of, say, histidine from the list is not simply due to the fact that the body is possessed of an efficient mechanism for conserving imidazole rings when necessary and that prolonged deprivation might not eventually result in undesirable effects The most we can say is that the food factors listed include those amino acids the absence of which in the diet results in undesirable effects almost immediately In addition to negative nitrogen balances absence of any of the food factors resulted in failure of appetite, increasing fatigue and nervous irritability, even when the subject was not aware that his diet was deficient Another point to remember is that these experiments were conducted on full grown men Whether other amino acids in addition to the eight listed are necessary for the normal growth of the infant is not known, but is a likely possibility

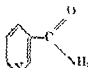
The portion of the particular amino acid which determines whether it is a food factor or not is, of course, the side chain since the alpha carbon itself together with its amino and carboxy substituents are common to all the amino acids The body is apparently unable to manufacture the branched hydrocarbon side chains of valine, leucine and isoleucine, while it is able to synthesize unbranched hydrocarbon side chains such as those in alanine, norleucine and proline Nor can it synthesize the benzene ring of phenylalanine The implication here is that if this non-synthesizable portion were supplied in a form similar to, but not identical with, the amino acid it could be utilized Thus, imidazole lactic acid (formula VI) or imidazole pyruvic acid (formula VII) can replace histidine (of which they are the hydroxy and keto analogs, respectively) in the diet of the rat Similarly, indole lactice acid (formula VIII) or indole pyruvic acid (formula IX) can replace tryptophane In each case, the non synthesizable heterocyclic ring is supplied and from it the complete amino acid can be formed by transamination (see Chapter 14) Since lysine does not take part in transamination reactions, however, the corresponding hydroxy or keto analogs of that amino acid are of no use to the animal organism

Given ample supplies of phenylalanine, the mammalian body is capable of adding the para hydroxy group to form tyrosine at a rate sufficient to fulfill its needs indefinitely even in the entire absence of dietary tyrosine The reverse is not true, so that of the two phenylalanine is the food factor and tyrosine is not Nor can the body synthesize tyrosine from any amino acid other than phenylalanine If, then to a diet containing only the amino acid food factors as protein source tyrosine is added, then the requirement of phenylalanine is decreased, since now only enough phenylalanine i needed for its own sake, none additional is necessary for the production of tyrosine This effect of added tyrosine is known as sparing action Cystine

deficiencies were noted without the biochemists of the day being able to determine the chemical nature of the food factors Early investigations led to the belief that one of the mysterious missing factors was an amine (and surely enough, the one then being studied thiamine, was an amine), so that the name of vitamine (i e , life amine) was given them Later it was found that not all "vitamines" are amines but the name had become too firmly embedded in the literature for any change to be made other than the minor one of dropping the final 'e' and thus mutilating the suffix out of a complete resemblance to the amine it often was not Because of the mystery that surrounded these trace substances at the beginning both medical men and the lay public took to regarding vitamins with something of awe and wonder Actually, the vitamins are no more mysterious than for instance, the amino acid lysine, and are food factors in the same sense that lysine is—the only difference being that the vitamins are needed in much smaller quantities

A vitamin is not always a distinct chemical individual Just as indole





X Nicotinic acid (niacin) XI Nicotinamide (niacinamide)

acetic acid can substitute for tryptophane since it supplies the needed indole ring, niacin can substitute for niacinamide Once the body is supplied with the needed carboxylated pyridine ring it can manage to form the amide from it The carboxyl group must exist on the ring however The body can not use pyridine itself as a niacinamide precursor When several closely related chemical substances can each function as a particular vitamin, the individuals are known as *vitamers* (the suffix being inspired by the more common term isomer) It is for this reason that vitamin assays whether chemical or microbiological must avoid being too specific, since the vitamin content of a particular foodstuff may be in the form of a mixture of vitamers and a test which is positive to one vitamer and negative to another will not give an accurate measure of its vitamin content

The system whereby vitamins are distinguished by letters of the alphabet also belongs to the early days of investigation when the chemical nature of these substances was unknown Two factors the absence of which give rise to two separate syndromes, could only be differentiated by being termed vitamin A and vitamin B This system of nomenclature is quite unsatisfactory Factors have been given letter designations and then identified as something known and the letter rendered unusable for future

which is not a food factor has a similar sparing action on methionine which is a food factor and which the body can use as a precursor for cystine

Rose has presented values for the minimum daily human requirement of each amino acid food factor where all other amino acids are amply supplied in the diet (see table 27). Rose states however, that the recommended daily intake in the formulation of diets should be twice the minimum daily requirement. Rose also investigated the possible utilization of D-amino acids by the body. He found that D methionine was as effective as the normal L form in the maintenance of nitrogen equilibrium, and that D phenylalanine could be partially utilized. The D-forms of the other amino acid food factors however, were entirely unutilizable by man

Other Organic Food Factors

The organic groupings other than amino acids associated with proteins in the form of prosthetic groups or coenzymes are in many cases synthesized from simpler components of the diet. Thus the nucleic acids are synthesized ultimately from glucose glycine and phosphate, mucopolysaccharides have glucose as their most important precursor and porphyrins are composed largely of glycine fragments. These substances just mentioned are needed by the body in relatively large quantities, and it would almost seem as though the organism dared not risk obtaining sufficient supplies of the ready made material or comparatively large fragments of it in the diet

In the case of many of the coenzymes however, only traces are needed and here the body tends to rely on the diet for certain essential portions. Thus in the case of DPN (see page 217) which is made up of a residue of adenine two of ribose two of phosphate and one of nicotinamide, the body reserves the ability to synthesize ribose and adenine from carbohydrate and amino acid food factors since both of these structures are needed in quantity in nucleic acids. Phosphorylated compounds are very widespread in the body and here likewise the organism always retains the ability to add phosphate to any organic substance capable of esterification. The nicotinamide however is a derivative of pyridine a ring system which does not occur in the body except in this and two other coenzymes none of which are needed in more than trace quantities. The cells of the human body have lost the ability to synthesize a structure needed in such small quantities and rely on dietary supplies. The simplest compound from which the cells can form the needed nicotinamide is nicotinic acid which is therefore the food factor. Niacin and niacinamide are common synonyms for nicotinic acid (formula X) and nicotinamide (formula XI)

An organic substance needed in traces in the diet for the proper functioning of the body is most frequently termed a *vitamin*. The name dates back to the turn of this century when the physiological effects of certain dietary

extracellular fluid, chlorine which is the major anion of plasma and extracellular fluid, potassium, which is the major cation of intracellular fluid, magnesium, which is present in both intracellular and extracellular fluid, and iron, which is a vital constituent of hemoglobin and the other heme catalysts

In addition to these, a number of minerals are essential to life in trace quantities due to their participation in the catalytic function of various enzymes or hormones Thus iodine is an essential part of thyroxine, zinc of the enzyme carbonic anhydrase, and cobalt of cyanocobalamine Copper, fluorine, and manganese are also essential in traces but their exact role in the body is as yet unknown It is these trace minerals which together with the vitamins, are included by the term, micronutrients In the case of all these mineral elements, from phosphorus on down, the food factors involved are the ions themselves Mineral metabolism is taken up in Chapter 15

HUMAN MALNUTRITION

Malnutrition results when the tissues receive inadequate amounts of any of the food factors The commonest reason for such a failure of food factor supply is their deficiency in the diet In the world as a whole, the most common by far of the varieties of malnutrition is *undernutrition*, a condition in which the food supply is insufficient to meet the energy needs of the body, so that a state of chronic starvation results Starvation will be discussed in Chapter 14

The United States is fortunate in that the average diet of its population is among the best in the world, and that undernutrition is relatively rare The U S Department of Agriculture has estimated that between 1943 and 1948 the per capita consumption of the various food factors has been above the quantity considered as 'recommended allowance", with the exception of calcium (7) Calcium was slightly below the recommended allowance, except in 1945 and 1946 when it was adequate This is not cause for unalloyed complacency, however This per capita figure is an average both in time and over a hundred fifty million people Individuals, groups, or even whole regions within the United States may be marginal or deficient in one or more of the food factors, or may have an adequate diet only during part of the year

In 1943, for instance, at which time the per capita consumption of all food factors other than calcium was considered sufficient, a survey of the diets of 71 individuals selected to obtain a distribution of age, sex, activity, and economic status showed only 7 per cent (all children) to be receiving a satisfactory level of all the nutrients analyzed, and only 79 per cent to be receiving at least two thirds of the recommended intake of all nutrients

designations. This happened to "Vitamin F", which was named, then abandoned, and is now thought to be identical with the unsaturated fatty acid food factor. Originally, the letters were awarded in alphabetical order, a system which extended consecutively up to and including "Vitamin H", but in other cases new vitamins were named according to the initial letter of the effect they brought about, as vitamin K which is the "Koagulations Vitamin" or vitamin P which is thought to be involved with capillary permeability. In addition, almost every lettered vitamin was eventually found to be multiple, not only in the sense of vitamerism, but in that some contained groups of factors possessing greatly different chemical natures and physiological functions. "Vitamin B" was particularly malignant in this respect. Since early separations involved extraction by different solvents under various conditions, what was called "Vitamin B" was actually a mixture of many water soluble, nitrogen-containing food factors. Various fractions were isolated from "Vitamin B", which were labeled "Vitamin B_1", "Vitamin B_2", and so on. The latest vitamin of major importance to be isolated is the cobalt containing "Vitamin B_{12}", which is also known as cyanocobalamine. Different workers, isolating the same factor, simultaneously bestowed different names on it. What some workers called "Vitamin B_2", for instance, was identical with what others called "Vitamin G". At the present time most of the trace organic food factors have been chemically identified and have received names. It is well to use the names rather than the letter designation wherever possible.

The individual vitamins are listed and discussed systematically in Chapter 19.

Mineral Food Factors

There must be at least one food factor containing each of the chemical elements necessary to life. Carbon, hydrogen, oxygen, nitrogen, and sulfur are supplied in large quantities by the carbohydrate, lipid, protein, and water of the diet. The remaining elements necessary to life are usually thought of nutritionally as *minerals*. They are distinguished from the elements we have been concerned with thus far in that they are available to the organism when ingested in their ionic forms.

Phosphorus represents an intermediate element. Although it can be utilized by the body when ingested as phosphate ion and is considered therefore one of the mineral elements of food, it is chiefly present in the diet as nucleic acid, phospholipid, phosphoprotein, and phosphorylated sugars and sugar derivatives. Other elements needed in comparatively large quantities are calcium, which with phosphate comprises the major portion of the teeth and bones, sodium, which is the major cation of plasma and

Destruction of vitamins in otherwise adequate diets during the cooking process either by heating for too long a period or by excessive soaking in water is a factor that must be taken into consideration. Ascorbic acid is particularly liable to destruction by heat; the B vitamins and the various minerals are liable to extraction in boiling. Less commonly, destruction of vitamins may proceed after ingestion. Thus ascorbic acid is reasonably stable only in quite acid solutions where the pH is 5 or less, and is oxidized at a rapidly increasing rate as its surroundings become more alkaline. In achlorhydric patients where the pH of the gastric juice is much higher than normal the amount of ascorbic acid available is seriously reduced.

The deleterious effect of prolonged cooking upon certain vitamins is not to be taken as an indication of the undesirability of cooking as a general rule. Cooking is nutritionally valuable for several reasons. Subjectively, it tends to improve the flavor and increase the palatability of foods. Objectively, it starts the process of hydrolysis of both proteins and starch so that cooked food is generally more easily digestible than raw food, and it also serves to kill bacteria and parasites present in the food item. As an example of the last very important item we may cite the well known danger of trichinosis infection following the ingestion of insufficiently cooked pork.

In some cases, particularly where the vitamins are concerned, the diet may not actually be as deficient as a simple analysis of the food stuffs may indicate. Certain food factors, notably vitamin K, cyanocobalamine, folic acid, and biotin, are produced by intestinal bacteria in amounts sufficient for human needs so that, strictly speaking, although these substances are true food factors and can not be synthesized within the human body, they need not be present in the human diet. In this respect, at least, the relationship of the intestinal bacteria to their human host is symbiotic rather than parasitic.

Nicotinic acid may be added to the list of vitamins formed by bacterial action in the intestines, provided sufficient tryptophane is present in the diet, tryptophane being the precursor out of which the intestinal flora synthesize the vitamin (17). Maize contains indoleacetic acid (formula XII) which by competitive inhibition interferes with the bacterial mechanism whereby tryptophane is utilized. The incidence of pellagra among populations using maize as a major component of the diet may therefore not be due so much to actual lack of nicotinic acid or its precursor, tryptophane, as to the presence of this *anti vitamin* (8).

Again, it is unrealistic to consider each food factor by itself. The metabolic interrelationships of carbohydrate, lipid, and protein are such that the need for any one of them is affected by the quantity of the other two in the diet. A good diet would not be merely one in which there was at least an adequate amount of each, but one in which the quantities of all three

(9) Jolliffe (7) states that

> a rough approximation would suggest that the prevalence of unsatisfactory diets is about 75 per cent in some groups and may reach 90 per cent. Even the prevalence of diets below bare subsistence levels providing only one fourth of the full allowances is dangerously frequent and exceeds by far the prevalence of many diseases which receive much publicity and generous public contributions for their study and amelioration.

The most important single cause of dietary inadequacy is of course economic. In general, high protein foods are more expensive than carbohydrate foods. Families which are forced to devise means for maintaining a food supply at the least possible expense almost unavoidably choose a diet which, even if adequate in its caloric content, is deficient in essential amino acids, the B vitamins, calcium, and often in other factors as well.

Where money is no object there are still the deleterious effects of artificial overselection of food items as a matter of taste, habit, carelessness or faddism. Radical reducing diets, particularly where not undertaken with medical supervision, are often dangerously deficient. Dangerous food habits which are much more widespread than the individual peculiarities just referred to and which are undesirable aspects of American diet as a whole are overconsumption of highly refined foods and, particularly, the immoderate use of sugar. The overrefinement of the grains used in bread making results in loss of those portions which contain the major part of the B vitamin content. This, fortunately, is being offset in recent years by the greater public awareness of the problem and by the use of vitamin concentrates in "enriched" flour.

Malnutrition may also be caused by conditions other than direct dietary deficiencies. A diet ordinarily adequate may become deficient if bodily requirements are increased, either for physiological reasons as in pregnancy and during lactation or for pathological reasons as under conditions of hyperthyroidism. Where the individual is normally engaged in strenuous physical labor, requirements of all nutrients, including the B vitamins, are sharply increased.

It is not to be forgotten, moreover, that ingestion does not automatically mean that the food constituents are available for use by the body. There is the hurdle of absorption yet to overcome. Chronic gastrointestinal disease by reducing the opportunity for absorption may bring about deficiency diseases even where the diet itself is adequate. At least one pathological condition, that of pernicious anemia, is brought about by the failure of the body to absorb cyanocobalamine, even though adequate quantities are always available in the intestine where it is formed through bacterial action (see Chapter 19).

Destruction of vitamins in otherwise adequate diets during the coo process either by heating for too long a period or by excessive soaki water, is a factor that must be taken into consideration Ascorbic ac particularly liable to destruction by heat the B vitamins and the va minerals are liable to extraction in boiling Less commonly destructi vitamins may proceed after ingestion Thus ascorbic acid is reason stable only in quite acid solutions where the pH is 5 or less and is oxi at a rapidly increasing rate as its surroundings become more alkalin achlorhydric patients where the pH of the gastric juice is much h than normal the amount of ascorbic acid available is seriously reduc

The deleterious effect of prolonged cooking upon certain vitamins i to be taken as an indication of the undesirability of cooking as a ge rule Cooking is nutritionally valuable for several reasons Subjecti it tends to improve the flavor and increase the palatability of foods jectively it starts the process of hydrolysis of both proteins and st so that cooked food is generally more easily digestible than raw food it also serves to kill bacteria and parasites present in the food item A example of the last very important item we may cite the well known da of trichinosis infection following the ingestion of insufficiently cooked p

In some cases, particularly where the vitamins are concerned, the may not actually be as deficient as a simple analysis of the food may indicate Certain food factors, notably vitamin B_{12} cyanocobalar folic acid and biotin are produced by intestinal bacteria in amounts ficient for human needs so that, strictly speaking, although these stances are true food factors and can not be synthesized within the hu body they need not be present in the human diet In this respect, at l the relationship of the intestinal bacteria to their human host is symb rather than parasitic

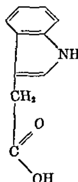
Nicotinic acid may be added to the list of vitamins formed by bact action in the intestines, provided sufficient tryptophane is present in diet, tryptophane being the precursor out of which the intestinal synthesize the vitamin (17) Maize contains indoleacetic acid (formula which by competitive inhibition interferes with the bacterial mecha whereby tryptophane is utilized The incidence of pellagra among po tions using maize as a major component of the diet may therefore n due so much to actual lack of nicotinic acid or its precursor, tryptop as to the presence of this anti-vitamin (8)

Again it is unrealistic to consider each food factor by itself The meta interrelationships of carbohydrate lipid, and protein are such that need for any one of them is affected by the quantity of the other tw the diet A good diet would not be merely one in which there was at an adequate amount of each, but one in which the quantities of all t

were well balanced Among the micronutrients, such interrelationships may also exist The tocopherols, by their activity in poising the oxidation reduction potential, increase the stability of other vitamins which would otherwise be subject to destruction by oxidation (4) Such an effect, where two substances together are more effective than the sum of each separately, is called *synergism*

Carbohydrate and Lipid

Some 50 to 60 per cent of the caloric intake of the average American is in the form of carbohydrate The percentage tends to be higher among the less prosperous groups A diet which is higher than average in carbohydrate need not necessarily be one which is deficient in other respects, although it is more apt to be so than one which is high in protein, par



XII Indoleacetic acid

ticularly where an excessive amount of the carbohydrate is in the form of highly refined grains and sugars

The function of both carbohydrate and fat in the body is to provide energy Carbohydrate, from carbohydrate or protein in the diet, is constantly being utilized by the various tissues of the body and is the ultimate power house which keeps the body machinery working Carbohydrate is stored in the animal body as glycogen, mainly in the liver and muscles but the total amount so stored is small in terms of calories being less than would be required to sustain normal activity a single day Soskin and Levine (18) set the total body carbohydrate of a hypothetical normal man with a body weight of 70 kgm , a muscle weight of 35 kgm , a liver weight of 1 8 kgm , and a blood plus extracellular fluid volume of 21 liters, to be 370 grams Muscle contains 0 7 per cent glycogen, giving a total of 245 grams in all the muscles Liver contains 6 per cent glycogen, giving a total of 108 grams The remaining 17 grams is in blood and extracellular fluid in the form of glucose This mass of carbohydrate has a caloric equiva

lent of 1517 Kcal, allowing 4 1 Kcal per gram, and is sufficient to maintain a human being engaged in a sedentary occupation for 13 hours Caloric reserves in excess of this are in the form of simple lipid which can be stored in almost indefinite quantities This so called depot fat takes up a smaller volume per calorie than does carbohydrate and is thus the most compact form for storing calories in anticipation of a long term need

Since carbohydrate can readily be converted into fat in the body, the amount of lipid in an adequate diet need not be high In fact, it need be present only in quantities sufficient to insure an adequate supply of fat soluble vitamins and, possibly of such unsaturated fatty acids as linoleic and arachidonic acids The amount should be increased where it is desirable to avoid the necessity of consuming too bulky a meal in order to obtain the necessary calories The latter qualification is of importance only where a person is engaged in an extremely active occupation or if, like the Eskimo, he is continuously exposed to cold environments

The nutritionally useful carbohydrates include the various starches, glycogen and their hydrolysis products, the dextrins, maltose and glucose, also fructose and galactose, together with their glucose containing disaccharide derivatives, sucrose and lactose Before absorption, the polysaccharides and disaccharides are hydrolyzed to monosaccharides which can be stored in the form of glycogen An important glucose-containing polysaccharide which is *not* directly available for human use is cellulose It is ingested in very appreciable quantities along with most plant food, particularly those of the leafy vegetables, but is excreted in the feces unchanged except for minor bacterial decomposition Its only function within the alimentary canal is the rather dubious one of supplying roughage"—that is, stimulating by its bulk and by the roughness of its texture the natural peristalsis of the intestine Indirectly, however, cellulose is of great importance to human nutrition since herbivorous animals possess alimentary canals of much greater capacity comparatively than those of carnivorous animals such as man, so that food retention is longer and intestinal bacteria have ample opportunity to hydrolyze cellulose to glucose Cattle are thus able to subsist on grass and hay and to convert these cheap items into expensive meat and dairy products Presumably, if the human possessed an alimentary canal of adequate capacity, he could utilize cellulose directly, but the current method of indirect utilization of cellulose is likely to remain widely popular

Obesity. The excessive intake of carbohydrate may be harmful in two ways By satisfying the appetite, it may reduce consumption of foods containing proteins and micronutrients thus leading to deficiency states in those respects Secondly, by increasing the over-all caloric intake it may induce obesity Obesity is almost invariably caused by eating more food

than is required In relatively few instances this situation is brought about by metabolic malfunction To reduce overweight it is necessary to restrict caloric intake to a point where it is below caloric expenditure This is an unavoidable consequence of simple arithmetic Weight reduction may be achieved either by reducing intake (dieting), or by increasing expenditure (exercise) Of the two the latter is by far the less satisfactory A sudden addiction to violent exercise, particularly in the case of a middle-aged or sedentary person may involve considerable and undesirable strain on the circulatory system Furthermore, increased exercise is accompanied by increased appetite with the all too frequent result that the net effect on weight loss is zero, or negative That this is not too surprising is demonstrated by the fact that the calories expended in climbing twenty flights of stairs, each ten feet high, may be restored by the consumption of one slice of bread and the calories expended by walking a mile, by the consumption of an ounce of cream Refraining from eating that slice of bread or that ounce of cream is obviously the easier method of accomplishing the result

In reducing caloric intake, the greatest danger is that the individual having spent happy years adding inches to his waistline, is suddenly determined to remove the accumulation in a matter of days While a fierce and uncontrolled regimen of lettuce and fat free crackers will not expose an obese person to the immediate danger of starvation, there is the very good chance that deficiencies in proteins and micronutrients may develop far more quickly than the obesity is abated

The exact dietary rules best qualified to effect weight reduction are not yet beyond dispute Soskin and Levin (18) recommend one gram of protein per day per kilogram of "ideal" body weight and see no harm in protein intakes in excess of this It goes without saying that protein in the diet must be such that at least minimal quantities of the various essential amino acids be supplied (see page 412) The use of vitamin concentrates and mineral supplements is necessary in drastic reducing diets

The relative proportions of lipids and carbohydrate intake in reducing diets is of importance A drastic reduction of "starchy" foods may be self defeating if lipids are not simultaneously restricted A diet relatively high in lipid and low in carbohydrate and protein will induce ketosis, so that however restricted the caloric intake, the carbohydrate and protein content should be high enough to prevent this Soskin and Levine suggest that the carbohydrate content of the reducing diet be two to three times (in calories) that of the lipid content Short range fluctuations of weight during dieting may result from changes in the water content of the body and in the first stages of dieting fat loss may often be obscured or exaggerated Once the desired weight has been attained the patient should

avoid reverting to his pre-diet food habits or in time the job will be all to do over again

Protein

Two aspects of protein malnutrition may be mentioned One is the general deficiency of protein i e ingestion of insufficient protein to maintain nitrogen balance (see Chapter 14) A second type of protein malnutrition lies in the deficiency of one or more of the amino acid food factors Each amino acid is slowly used up in normal metabolism, and either itself or a precursor must be supplied in the diet Since the amino acid food factors such as lysine or tryptophane have no precursors among the other amino acids they must be supplied as such or nitrogen equilibrium will not be maintained regardless of the quantity of other amino acids consumed Amounts of amino acids in excess of requirement will be metabolized and the nitrogen eventually excreted, chiefly as urea (see page 535)

The minimum amount of protein needed to maintain nitrogen equilibrium (and hence the minimum amount necessary for an adequate diet) is still a matter of controversy Early investigations, before the significance of the amino acid food factors was known, yielded results that were naturally conflicting since various proteins would yield various minima depending upon the weight of protein which would contain an adequate amount of the amino acid food factor in which it was poorest In the case of proteins such as gelatin or zein the weight required would approach the infinite. Taking the amino acid food factors into account, Newburgh maintains that 60 grams of protein per day will suffice to maintain nitrogen balance (14) As for the amino acid food factors themselves Rose has calculated the minimum intake required for nitrogen balance in the adult male, *where there is an adequate dietary supply of the other amino acids*, as 6 35 grams (see table 27)

Protein requirement is increased in various pathological conditions There is serious protein loss in cases of hemorrhage since one liter of blood contains 140 grams of hemoglobin and 60 grams of plasma protein, or a total of 200 grams of protein The normal rate of tissue protein breakdown is accelerated during infection, and after various injuries and after operations (21) Proteins may be lost in exudates following burns or wounds, or via the urine in albuminuria In the former case, losses may amount to 50 grams per day or even more and in the latter, to 25 grams Protein deficiencies may also result from metabolic disturbances which reduce the body's ability to synthesize protein from amino acids

The most obvious result of chronic protein malnutrition is loss in weight This is to be distinguished from loss in weight due to uncomplicated caloric

deficiency in that the loss is represented not by decline in depot fat but by actual wasting of functional tissue Protein deficiency is usually accompanied by disturbances of the water balance in part due to the lowered osmotic pressure following loss of serum albumin Water tends to be retained while tissues may waste up to 25 per cent of their original weight, the consequent disproportion of water content resulting in *nutritional edema* The bloated abdomens of famine victims are characteristic Restoration of a diet adequate in protein, by leading to an initial loss of excess water, may actually result in a decrease in weight at first

Daily Food Factor Requirements

The recommended daily dietary allowances of the Food and Nutrition Board of the National Research Council, as of 1948, are included in table 28 (10) A diet which is adequate for the factors listed in the table will provide adequate quantities of other minerals and vitamins Thus a diet adequate in calcium is almost certainly adequate in phosphorus, while one which will supply the body with the iron it needs will also supply it with copper Some precautions in the use of the table must be pointed out

Calories. The figures given relate to men, women, and children of given weights In assigning calorie allowances to individuals allowance must be made for weight differences (due to differences in body build, that is, and not to differences due to obesity or past history of undernutrition) In the case of children particularly, the figures are based on average requirements for the middle age of the various age ranges In children of the same age, allowance must be made for differences in size and activity The figure given for women in the latter half of pregnancy is for a sedentary woman In general, calorie requirements during late pregnancy should be 20 per cent higher than that for a non pregnant woman of the same size and activity. This rule of thumb assumes the woman to be of normal weight at the time of pregnancy The pregnant woman should be allowed to gain 20 to 25 pounds above her ideal normal weight for height, age, and build This implies that the underweight woman should be allowed to gain more and the overweight woman should be carefully controlled The subdivision of calories into carbohydrates and lipids is flexible It is recommended that 20 to 25 per cent of the total calories be taken as fat in the case of sedentary persons, 30 to 35 per cent in the case of active persons

Individual food factors. The Food and Nutrition Board recommends that one per cent of the total calories be taken in the form of the unsaturated fatty acid food factors The protein requirement is predicated, of course, on the presence of ample supplies of each of the amino acid food factors in the diet The iron requirement for adult males, despite the figure given in the table, is probably close to zero except where it is necessary to replace

losses due to hemorrhage Iodine requirements by the body are small about 0 15 to 0 30 mgm per day In some areas of the world where the soil is poor in iodine and seafood is unavailable endemic iodine deficiency may exist a condition especially troublesome in adolescence and pregnancy The deficiency can be easily corrected by the use of iodized salt Supple

TABLE 28

Recommended daily dietary allowances—revised 1948 Food and Nutrition Board National Research Council

	CALORIES	PROTEIN	CALCIUM	IRON	VITAMIN A	THIAMINE	RIBOFLAVIN	NIACIN (NICOTINIC ACID)	ASCORBIC ACID	VITAMIN D
		gm	gm	mgm	I U	mgm	mgm	mgm	mgm	I U
Man (70 kgm)										
Sedentary	2 400	70	1 0	12	5 000	1 2	1 8	12	75	—
Physically active	3 000	70	1 0	12	5 000	1 5	1 8	15	75	—
With heavy work	4 500	70	1 0	12	5 000	1 8	1 8	18	75	
Woman (56 kgm)										
Sedentary	2 000	60	1 0	12	5 000	1 0	1 5	10	70	—
Moderately active	2 400	60	1 0	12	5 000	1 2	1 5	12	70	—
Very active	3 000	60	1 0	12	5 000	1 5	1 5	15	70	—
Pregnancy (latter half)	2 400	85	1 5	15	6 000	1 5	2 5	15	100	400
Lactation	3 000	100	2 0	15	8 000	1 5	3 0	15	150	400
Children up to 12 yrs										
Under 1 yr	110/kgm	3 5/kgm	1 0	6	1 500	0 4	0 6	4	30	400
1-3 yrs (12 kgm)	1 200	40	1 0	7	2 000	0 6	0 9	6	35	400
4-6 yrs (19 kgm)	1 600	50	1 0	8	2 500	0 8	1 2	8	50	400
7-9 yrs (26 kgm)	2 000	60	1 0	10	3 500	1 0	1 5	10	60	400
10-12 yrs (35 kgm)	2 500	70	1 2	12	4 500	1 2	1 8	12	75	400
Children over 12 yrs										
Girls 13 15 yrs (49 kgm)	2 600	80	1 3	15	5 000	1 3	2 0	13	80	400
16–20 yrs (64 kgm)	2 400	75	1 0	15	5 000	1 2	1 8	12	80	400
Boys 13 15 yrs (49 kgm)	3 200	85	1 4	15	5 000	1 5	2 0	15	90	400
16–20 yrs (64 kgm)	3 800	100	1 4	15	6 000	1 7	2 5	17	100	400

mental vitamin K is needed for the pregnant woman immediately before parturition or for the infant immediately after birth in order that the infant may have a sufficient supply of this blood clotting factor until such time as it has accumulated vitamin synthesizing intestinal flora of its own. Water intake is quite variable and may be allowed *ad libitum* since sensations of thirst usually serve as adequate guides to intake except for infants and sick persons. The additional supply of the various food factors, other than ascorbic acid and vitamin D, required by the pregnant or lactating woman can be supplied by adding an additional pint of milk to the daily diet—that is, a total of a quart and half per day.

In summarizing, it should be emphasized that with the exception of vitamin D the recommended allowances of the Food and Nutrition Board can be supplied with a varied diet of common foods. There are many alternate sources in foodstuffs for any of the food factors, except calcium and ascorbic acid. An adequate calcium supply virtually requires the inclusion of milk or cheese in the diet, while to obtain ascorbic acid, citrus fruits and tomatoes have no equals. Where vitamin D is needed the use of enriched milk or fish liver oil preparations is advisable.

For detailed information on specialized diets in old age, illness and disease, the student is referred to the Handbook of Nutrition (19, 11). The special case of infant feeding will be taken up later.

FOODS

Milk

Milk is a food that millions of years of evolution have developed as the most nearly ideal food for the mammalian young. Its most important proteins, *casein* and *lactoglobulin*, are typical complete proteins supplying an adequate quantity for growth of each of the amino acid food factors. With the exception of iron and vitamin D, milk is a rich source for all the other food factors as well.

The characteristic carbohydrate of milk is lactose, a disaccharide containing glucose and galactose. The purpose of the galactose is not yet understood, and there is no reason to suspect that it is a food factor. The presence of galacto lipids in the brain and nervous system may or may not be important in this connection. The lipid content of milk is distributed through the aqueous substratum as a coarse emulsion which will separate out on standing. Homogenization, by reducing the fat particles in milk to smaller dimensions, forms a permanent emulsion. While homogenization may alter the texture and taste of the milk to a small extent and will insure an even distribution of the various food factors throughout the milk it does not alter the nutritional value of the milk as a whole.

Many popular food items are milk derivatives, or as they are better

known dairy products *Cheese* is the product made from the separated curd obtained by coagulating the protein of milk The coagulation is accomplished by the use of rennet or other suitable enzymes, lactic fermentation, or a combination of the two There are numerous varieties of cheese, depending upon the details of the procedure with respect to heat, pressure, ferments molds, or seasoning used Nevertheless, nutritionally all cheeses have this in common they are essentially concentrates of milk protein and, to a varying extent of milk fat A pound of cheddar cheese represents the casein and fat of a gallon of average milk Cheese is thus an excellent protein food, on a par with meat, and is a good source of phosphorus, calcium, and vitamin A (the last accompanying the fat content of cheese)

Cream is that portion of milk which rises on standing Since fat is lighter than water, that portion is a milk fat concentrate The fat content of cream varies according to the method of separation and is usually less than 35 per cent The vitamin A of milk is concentrated along with the fat The fat poor portion of milk remaining after cream has been removed is *skim milk*, which has considerable food value despite the unfavorable connotations attached to the name It contains in large measure the proteins and minerals of milk and is a valuable addition to the diet of those attempting to reduce

By churning the fat globules of cream fresh or fermented, can be made to coalesce, forming *butter* and leaving behind the aqueous portions of cream in the form of *buttermilk* Butter is a still more concentrated form of milk fat than is cream the fat content being over 80 per cent and is a correspondingly richer source of vitamin A Milk is also often used in making oleomargarine, a high fat product closely resembling butter in taste and texture and differing in that the source of its fat is not primarily milk fat but other less expensive fats Oleomargarine does not naturally have the vitamin content of butter These are customarily added, however to table oleomargarine

Infant feeding. While milk and its derivatives are all important adjuncts to the diet at all ages, it forms the major portion of the diet during the first year of life and is of particular interest in that connection While human milk is the particular variety used for the nutrition of infants in a state of nature, under the complex conditions of our own society cow's milk is frequently used instead The variation in chemical composition between human milk and cow's milk is not such as to prevent the latter from being an excellent dietary item in the adult To the infant however, the differences are sufficient to make necessary definite modifications of the cow's milk intended for the infant stomach These differences are most marked with respect to the concentration of protein, carbohydrate, and minerals

mental vitamin K is needed for the pregnant woman immediately before parturition or for the infant immediately after birth in order that the infant may have a sufficient supply of this blood clotting factor until such time as it has accumulated vitamin synthesizing intestinal flora of its own Water intake is quite variable and may be allowed *ad libitum* since sensations of thirst usually serve as adequate guides to intake except for infants and sick persons The additional supply of the various food factors, other than ascorbic acid and vitamin D, required by the pregnant or lactating woman can be supplied by adding an additional pint of milk to the daily diet—that is, a total of a quart and half per day

In summarizing, it should be emphasized that with the exception of vitamin D the recommended allowances of the Food and Nutrition Board can be supplied with a varied diet of common foods There are many alternate sources in foodstuffs for any of the food factors, except calcium and ascorbic acid An adequate calcium supply virtually requires the inclusion of milk or cheese in the diet, while to obtain ascorbic acid, citrus fruits and tomatoes have no equals Where vitamin D is needed the use of enriched milk or fish liver oil preparations is advisable

For detailed information on specialized diets in old age, illness and disease, the student is referred to the Handbook of Nutrition (10 11) The special case of infant feeding will be taken up later

FOODS

Milk

Milk is a food that millions of years of evolution have developed as the most nearly ideal food for the mammalian young Its most important proteins, *casein* and *lactoglobulin*, are typical complete proteins supplying an adequate quantity for growth of each of the amino acid food factors With the exception of iron and vitamin D, milk is a rich source for all the other food factors as well

The characteristic carbohydrate of milk is lactose, a disaccharide containing glucose and galactose The purpose of the galactose is not yet understood, and there is no reason to suspect that it is a food factor The presence of galacto lipids in the brain and nervous system may or may not be important in this connection The lipid content of milk is distributed through the aqueous substratum as a coarse emulsion which will separate out on standing Homogenization, by reducing the fat particles in milk to smaller dimensions, forms a permanent emulsion While homogenization may alter the texture and taste of the milk to a small extent and will insure an even distribution of the various food factors throughout the milk it does not alter the nutritional value of the milk as a whole

Many popular food items are milk derivatives, or as they are better

known dairy products *Cheese* is the product made from the separated curd obtained by coagulating the protein of milk The coagulation is accomplished by the use of rennet or other suitable enzymes, lactic fermentation, or a combination of the two There are numerous varieties of cheese, depending upon the details of the procedure with respect to heat, pressure, ferments molds, or seasoning used Nevertheless, nutritionally all cheeses have this in common they are essentially concentrates of milk protein and, to a varying extent of milk fat A pound of cheddar cheese represents the casein and fat of a gallon of average milk Cheese is thus an excellent protein food, on a par with meat, and is a good source of phosphorus, calcium, and vitamin A (the last accompanying the fat content of cheese)

Cream is that portion of milk which rises on standing Since fat is lighter than water, that portion is milk fat concentrate The fat content of cream varies according to the method of separation and is usually less than 35 per cent The vitamin A of milk is concentrated along with the fat The fat poor portion of milk remaining after cream has been removed is *skim milk*, which has considerable food value despite the unfavorable connotations attached to the name It contains in large measure, the proteins and minerals of milk and is a valuable addition to the diet of those attempting to reduce

By churning, the fat globules of cream fresh or fermented, can be made to coalesce, forming *butter* and leaving behind the aqueous portions of cream in the form of *buttermilk* Butter is a still more concentrated form of milk fat than is cream the fat content being over 80 per cent and is a correspondingly richer source of vitamin A Milk is also often used in making oleomargarine, a high fat product closely resembling butter in taste and texture, and differing in that the source of its fat is not primarily milk fat but other less expensive fats Oleomargarine does not naturally have the vitamin content of butter These are customarily added, however, to table oleomargarine

Infant feeding. While milk and its derivatives are all important adjuncts to the diet at all ages, it forms the major portion of the diet during the first year of life and is of particular interest in that connection While human milk is the particular variety used for the nutrition of infants in a state of nature, under the complex conditions of our own society *cow's milk is frequently used instead The variation in chemical composition* between human milk and cow's milk is not such as to prevent the latter from being an excellent dietary item in the adult. To the infant however, the differences are sufficient to make necessary definite modifications of the cow's milk intended for the infant stomach These differences are most marked with respect to the concentration of protein, carbohydrate, and minerals

mental vitamin K is needed for the pregnant woman immediately before parturition or for the infant immediately after birth in order that the infant may have a sufficient supply of this blood clotting factor until such time as it has accumulated vitamin synthesizing intestinal flora of its own. Water intake is quite variable and may be allowed *ad libitum* since sensations of thirst usually serve as adequate guides to intake except for infants and sick persons. The additional supply of the various food factors other than ascorbic acid and vitamin D, required by the pregnant or lactating woman can be supplied by adding an additional pint of milk to the daily diet—that is, a total of a quart and half per day.

In summarizing, it should be emphasized that with the exception of vitamin D the recommended allowances of the Food and Nutrition Board can be supplied with a varied diet of common foods. There are many alternate sources in foodstuffs for any of the food factors, except calcium and ascorbic acid. An adequate calcium supply virtually requires the inclusion of milk or cheese in the diet, while to obtain ascorbic acid citrus fruits and tomatoes have no equals. Where vitamin D is needed, the use of enriched milk or fish liver oil preparations is advisable.

For detailed information on specialized diets in old age, illness, and disease, the student is referred to the Handbook of Nutrition (19, 11). The special case of infant feeding will be taken up later.

FOODS

Milk

Milk is a food that millions of years of evolution have developed as the most nearly ideal food for the mammalian young. Its most important proteins, *casein* and *lactoglobulin*, are typical complete proteins supplying an adequate quantity for growth of each of the amino acid food factors. With the exception of iron and vitamin D, milk is a rich source for all the other food factors as well.

The characteristic carbohydrate of milk is lactose, a disaccharide containing glucose and galactose. The purpose of the galactose is not yet understood, and there is no reason to suspect that it is a food factor. The presence of galacto lipids in the brain and nervous system may or may not be important in this connection. The lipid content of milk is distributed through the aqueous substratum as a coarse emulsion which will separate out on standing. Homogenization by reducing the fat particles in milk to smaller dimensions, forms a permanent emulsion. While homogenization may alter the texture and taste of the milk to a small extent and will insure an even distribution of the various food factors throughout the milk, it does not alter the nutritional value of the milk as a whole.

Many popular food items are milk derivatives, or as they are better

known dairy products *Cheese* is the product made from the separated curd obtained by coagulating the protein of milk The coagulation is accomplished by the use of rennet or other suitable enzymes, lactic fermentation, or a combination of the two There are numerous varieties of cheese, depending upon the details of the procedure with respect to heat, pressure, ferments molds, or seasoning used Nevertheless, nutritionally all cheeses have this in common they are essentially concentrates of milk protein and, to a varying extent of milk fat A pound of cheddar cheese represents the casein and fat of a gallon of average milk Cheese is thus an excellent protein food on a par with meat, and is a good source of phosphorus, calcium and vitamin A (the last accompanying the fat content of cheese)

Cream is that portion of milk which rises on standing Since fat is lighter than water, that portion is a milk fat concentrate The fat content of cream varies according to the method of separation and is usually less than 35 per cent The vitamin A of milk is concentrated along with the fat The fat poor portion of milk remaining after cream has been removed is *skim milk*, which has considerable food value despite the unfavorable connotations attached to the name It contains in large measure the proteins and minerals of milk and is a valuable addition to the diet of those attempting to reduce

By churning the fat globules of cream fresh or fermented, can be made to coalesce, forming *butter* and leaving behind the aqueous portions of cream in the form of *buttermilk* Butter is a still more concentrated form of milk fat than is cream the fat content being over 80 per cent and is a correspondingly richer source of vitamin A Milk is also often used in making oleomargarine, a high fat product closely resembling butter in taste and texture and differing in that the source of its fat is not primarily milk fat but other less expensive fats Oleomargarine does not naturally have the vitamin content of butter These are customarily added, however to table oleomargarine

Infant feeding While milk and its derivatives are all important adjuncts to the diet at all ages, it forms the major portion of the diet during the first year of life and is of particular interest in that connection While human milk is the particular variety used for the nutrition of infants in a state of nature, under the complex conditions of our own society cow s milk is frequently used instead The variation in chemical composition between human milk and cow s milk is not such as to prevent the latter from being an excellent dietary item in the adult To the infant however the differences are sufficient to make necessary definite modifications of the cow s milk intended for the infant stomach These differences are most marked with respect to the concentration of protein, carbohydrate, and minerals

palmitic acid and stearic acid and a smaller proportion of oleic acid. Here, too, the difference is negligible nutritionally but again the more saturated glycerides are the slower to digest. Human milk is less than half as rich in the lower fatty acids as cow's milk and is particularly poorer in butyric acid. The fat globules of cow's milk are considerably larger than those of human milk.

Colostrum is secreted by the mammary gland shortly before parturition and for a few days thereafter. It is comparatively poor in fat—about two thirds that of ordinary milk—but has twice the protein content of milk. The additional protein is a globulin which appears identical with the gamma globulin of plasma. Protective antibodies representing the immunities developed by the mother are contained in this globulin fraction.

The two food factors in which both cow's milk and human milk are deficient are iron and vitamin D. The deficiency of iron is not serious for the baby since he is born with an iron reserve sufficient for about six months, by which time there will be a dietary supplement of such iron sources as egg yolk and cereal preparations. The vitamin D deficiency can be made up in either of two ways, exposure of the child to sunlight or the use of vitamin D as an addition to milk. Cow's milk can be enriched either by the direct addition of vitamin D preparations, by the feeding of such preparations to the cow, or by irradiating (exposing to sunlight or ultra violet light), the cow, the milk, or the fodder. The action of the sunlight is to photo oxidize steroid precursors in the fodder, milk, or skin to compounds with vitamin D activity.

Bottle fed babies are apt to show an undesirably low level of blood ascorbic acid within ten days after birth. Supplementary ascorbic acid in the form of orange juice, as a rule, is therefore desirable at a very early age. An ounce of orange juice a day is recommended from the first week on increasing to two ounces a day at three months of age. The premature

Eggs

Where milk is the specialized and complete food of the young mammal, the egg of the bird represents the specialized and complete food for the developing bird embryo. The egg yolk contains most of the egg's nutritional value. The most important protein of egg yolk is ovovitellin, which, like the casein of milk, is a phosphoprotein and a good source of all the amino acid food factors. *Of these factors, it has only one third the valine content* of casein and is about 25 per cent poorer in lysine.

The lipid content of egg yolk is high, comprising nearly one third of the

The protein concentration of cow's milk is about three times that of human milk, being 3 to 4 per cent in the former as compared with 1 to 1 5 per cent in the latter This is not surprising since the protein content of a particular variety of milk would be adapted to meet the growth requirements of the young of that species A calf doubles its birth weight in fifty days, while a baby requires one hundred eighty days to do the same Protein must be supplied in larger quantities to the former The amino acid distribution is, however very similar in cow's milk and human milk the most notable difference being the comparative poverty of cow's milk in cystine which, however, is not one of the amino acid food factors

The concentration of lactose in human milk is about 50 per cent higher than in cow's milk, the figures being 7 0 to 7 5 per cent and 4 5 to 4 9 per cent, respectively The ash of human milk is less than one third that of cow's milk, 0 2 per cent as compared with 0 7 per cent The distribution of individual minerals is similar in the two varieties of milk, except that human milk, although deficient in iron, is not quite as deficient as cow's milk These differences between human milk and cow's milk make it necessary to dilute cow s milk intended for infant use with about an equal amount of water The deficiency of sugar in cow's milk, accentuated by this dilution, is made up for by the addition of lactose or products of starch hydrolysis, including dextrins, maltose, and glucose The protein and ash content of this diluted milk is still higher than that of an equivalent volume of human milk by some 50 per cent This means that bottle fed babies store greater quantities of nitrogen and calcium than do breast fed babies The nitrogen retention particularly, results in a muscle mass approximately 25 per cent greater in the bottle fed babies The breast fed baby seems to be at no particular disadvantage, however, because of its lesser muscle mass On the other hand bottle fed babies whose formulas are diluted so as to yield only the protein concentration of human milk show poorer tissue turgor and poorer motor development

This is one indication that no matter how we attempt to make the proportions of the various food constituents in human and cow's milk identical, certain finer differences remain Thus in cow's milk the two most important proteins, casein and lactoglobulin, occur in approximately a 6 1 ratio whereas in human milk the corresponding ratio is about 2 3 (6) While the two proteins are both complete, casein forms a curd when subjected to the action of the gastric juices The result is that cow's milk is digested more slowly than human milk This is reflected in the fact that the feces of a bottle fed baby are generally more abundant and of a harder consistency than are those of a breast fed baby

Furthermore, cow's milk while possessing a fat concentration (about 4 per cent) similar to that of human milk, contains a larger proportion of

source of the B vitamins, particularly thiamine and riboflavin Lean pork, for instance, is one of the richest natural sources of thiamine

Special mention should be made of the glandular organs of the animals usually used for meat—the liver in particular The liver is the chemical factory of the body and is therefore particularly rich in enzymes, and consequently in coenzymes Since the B vitamins occur in the body as parts of coenzymes, liver is rich in these food factors, particularly in riboflavin Fresh liver is the food richest in riboflavin The fat soluble vitamins seem to be stored in the liver This is particularly true in the case of the vitamins A and D Storage can take place to a fantastic degree where the diet is rich in these vitamins Liver also possesses small but significant amounts of ascorbic acid The use of liver extracts in combating pernicious anemia is due to its content of cyanocobalamine

Fish and seafood in general closely resemble ordinary meats as far as the quantity and quality of the proteins are concerned The livers of certain fish such as cod and halibut, while not usually eaten directly, are the richest known natural sources of the vitamins A and D Fish liver oils are used in infant dietaries for the prevention of rickets, but are not preferable to vitamin-enriched milk, since the latter can be fed to the infant from the start whereas fish oils are not usually prescribed until the second month of life Seafood differs from ordinary meats in being higher in mineral content The most significant item here is its high iodine content Seafood generally has ten times the concentration of iodine of land food Since iodine is necessary in the biosynthesis of thyroxine the populations of regions where the iodine content of the soils and consequently of the land plants may be negligible often suffer from endemic goiter Such problems have been solved by small quantities of iodine in city reservoirs or by the use of iodized salt, these problems do not appear at all where seafood is a significant portion of the diet Salt water fish, as would be expected, are richer in minerals in general and iodine in particular than are fresh water fish

Grains

Most grains or cereals are seeds produced by members of the grass family If any foods may be considered the basic substratum of the human diet, it is these, and there is certainly no quarrel with the proverbial definition of bread as the "staff of life" Grains owe their importance to several factors They are relatively cheap and available low in water content, and may be stored over periods of time without spoiling, a fact not true of any of the animal foods so far discussed In the United States, cereal products account for more than one fourth of the total calorie intake, and of the cereals wheat products constitute 75 per cent In the world as a whole,

whole Most of the lipid is in the form of phospholipids (mainly lecithin) and cholesterol With respect to both protein and lipid, egg yolk approximates meat in chemical composition As far as minerals and vitamins are concerned, egg yolk is superior to meat and approaches, and in some respects surpasses milk in value Although calcium is only half as concentrated as in milk, eggs are twice as rich in phosphorus because of their phospholipid content One great advantage eggs have over milk lies in their content of iron and copper, for both of which egg yolk is one of the richest natural sources being nearly three times as rich in iron as lean beef and about twice as rich in copper Egg yolk is an excellent source for vitamin A and for the B vitamins It contains significant quantities of vitamin D, but is a poor source of ascorbic acid

Egg white is a 10 per cent colloidal solution of egg albumin, also known as ovalbumin, with small amounts of mucoprotein accompanying it Because of the comparative lack of other materials in egg white, egg albumin has been one of the proteins most investigated in the laboratory and it is significant that the German word for protein is *Eiweiss* Egg albumin is a nutritionally complete protein Raw egg white contains the protein *avidin*, which is capable of forming a stable complex with biotin, the complex possessing none of biotin s food factor properties The feeding of large amounts of raw egg white or of avidin to laboratory animals can produce a biotin deficiency The biotin inhibiting properties of egg white are destroyed on cooking

Meat

The chemical composition and nutritive value of meats varies only slightly among the more common sources in the American diet, and what is said here will hold for beef, veal, mutton, lamb, pork, and poultry

Fresh lean meat, i e, muscle tissue is about one fifth protein, mostly myosin Myosin is a complete protein which is nutritionally on a par with the proteins of milk and eggs The lipid content of meat varies with the cut and with the animal source, but this is not a vital factor except in the case of those on reducing diets This variable lipid content is of course the simple lipid of the depot fat The carbohydrate content of lean meat is low and confined largely to the muscle glycogen

Lean meat is poor in calcium, containing only one tenth the calcium concentration of milk, but is about as rich a source of phosphorus as is egg yolk Lean meat like eggs and unlike milk, is a good source for iron and copper and is low in vitamin C In addition it is poor in vitamins A and D Nor are these last named fat soluble vitamins found to any appreciable extent in the depot fat of animals Lean meat, however, is a good

read contributes to its vitamin content, as does the practice of manu
ıcturing whole wheat bread in which germ and bran are included In
cent years enrichment with various vitamins has become common

Rice contains less protein (8 per cent as compared with 10 per cent) and
ore carbohydrate than wheat Rice is therefore somewhat less suited as a
asic diet than is wheat The bran layers contain most of the thiamin
ɔntent of rice, but again whole rice does not keep as well as does polished
ce so that the economic pressures in favor of the latter are sometimes
verwhelming Of historical interest is the fact that the first avitaminosis
efinitely characterized and cured was beriberi, found to occur among
ast Indians subsisting on a diet composed largely of white (polished)
ce and cured by the addition of brown (unpolished) rice

Maize differs from wheat in containing an appreciable (4 per cent) quan
ty of lipid material From this corn oil is derived It is an unsatisfactory
od in itself since its proteins, while equal quantitatively to those of wheat,
re far less complete It is also much poorer than wheat in the B vitamins
) that among the lower economic strata of the southern part of the United
tates where the diet is composed in great part of cornbread and hominy
rits pellagra has long been endemic

ruits and Vegetables

Fruits with the exception of the banana contain no starch when fully
pened and most of the carbohydrate is in the form of utilizable sugars
he mineral content is generally lower than in vegetables Fruits are char
terized first by being among the richest natural sources of free mono-
ıccharides (usually glucose or fructose) and of sucrose which, together
ith their content of organic acids (usually malic acid or citric acid) and
lated compounds that contribute to that elusive element we call flavor
ake them very pleasant eating Secondly certain fruits represent our
ıost valuable sources of ascorbic acid The juices of orange lemon, grape
 uit, pineapple and tomato are recognized as excellent sources of that
ɔod factor Orange juice is for this reason commonly added to the infant
iet at a very early age

Tomato juice is a rich source of provitamin A (see page 706), while pine
pple and orange juices are fair sources Fruits are poor in the B vitamins
ı general and in minerals Certain fruits such as olives and nuts are re
ıarkable and useful for their high lipid content

Vegetables, particularly the leafy vegetables are second only to fruits as
ources of ascorbic acid and are superior to fruits as sources of the B vita
ıins provitamin A, and minerals The importance of leafy vegetables, in
eneral, can however be overemphasized With a few exceptions they are
ery deficient in protein, and the caloric content of leafy vegetables is

rice is even more important than wheat since the vast populations of the Orient subsist to a large extent upon that grain Other important grains are maize oats barley rye and millet

The wheat kernel consists of three parts (1) an outer coat of *bran* which comprises 12 to 15 per cent of the whole (2) the *germ* which is the actual embryo plant and forms 2 to 3 per cent of the whole and (3) the *endosperm* which forms the remaining major portion of the kernel and represents the initial food supply of the young plant In the preparation of white flour the bran and germ are discarded in the milling process so that the flour is essentially finely ground endosperm Such flour is poor in protein as compared with meat both quantitatively and qualitatively In amount protein comprises 10 per cent of wheat flour as compared with 20 per cent in meat or eggs In quality the protein of wheat is less well endowed with amino acid food factors The glutenin of wheat is complete but gliadin is partially incomplete About 75 per cent of wheat flour is carbohydrate in the form of starch The lipid content is negligible

Wheat germ is ordinarily discarded in the milling process largely because its relatively high percentage of oil results in a tendency to become rancid and thus interferes with the keeping qualities of flour This is unfortunate since it is richer in protein minerals and vitamins than is the endosperm Because of the fact that it forms such a small portion of the whole kernel its contribution to the protein content is negligible but in the case of the micronutrients it is most important It is particularly rich in the B complex Thus whole wheat flour which contains wheat germ is over four times as rich in thiamin as refined flour despite the fact that in terms of mass the germ contributes only some 3 per cent of the flour Ascorbic acid is absent in wheat as are vitamins A and D

Wheat in common with most plant foods is rich in minerals with the exception of sodium Plants in general are potassium rich and sodium poor as compared with animals so that man and herbivorous animals are forced to supplement their diet with sodium chloride Mineral content is most concentrated in the bran which is discarded in the usual milling process Some two thirds of the carbohydrates of bran are indigestible pentosans so that bran is much used as roughage in the home treatment of constipation While undoubtedly often efficacious, the rough fibrous nature of bran makes it unfit for use in large quantities without medical supervision The irritating effect of bran may seriously aggravate disorders of the alimentary canal

Bread while composed mostly of wheat flour—at least in the United States possesses additives which affect its nutritive value usually for the better Milk is frequently used in the preparation of bread the nutritional value of this practice needing no comment The use of yeast in preparing

bread contributes to its vitamin content as does the practice of manufacturing whole wheat bread in which germ and bran are included In recent years, enrichment with various vitamins has become common

Rice contains less prot(8 per cent as compared with 10 per cent) and more carbohydrate than heat Rice is therefore somewhat less suited as a basic diet than is wheat The bran layers contain most of the thiamin content of rice but agai hole rice does not keep as well a does polished rice so that the econom pressures in fa r of the latter are sometimes overwhelming Of histori al interest is the fact that the first avitaminosis definitely characterized d cured was beriberi found to occur among East Indians subsisting a diet composed largely of white (polished) rice and cured by the addition of brow unpolished) rice

Maize differs from wh at in ntaining a appreciable (4 per cent) quantity of lipid material Fr m th corn oil derived It is an unsatisfactory food in itself since its pr ins while equal antitatively to those of wheat are far less complete It also much poorer than wheat in the B vitamins so that among the lower economic strata of he southern part of the United States where the diet omposed in great part of cornbread and hominy grits pellagra has long been endemic

Fruits and Vegetables

Fruits with the exception f the banana contain no starch when fully ripened and most of the carbohydrate is in the form of utilizable sugars The mineral content is generally lower than in vegetables Fruits are characterized first by being among the richest natural sources of free monosaccharides (usually glucose r fructose) and of sucrose which together with their content of organic acids (usually malic acid or citric acid) and related compounds that contribute to that elusive element we call flavor make them very pleasant eating Secondly certain fruits represent our most valuable sources of ascorbic acid The juices of orange lemon grapefruit, pineapple and tomato are recognized as excellent sources of that food factor Orange juice is for this reason commonly added to the infant diet at a very early age

Tomato juice is a rich source of provitamin A (see page 706) while pineapple and orange juices are fair sources Fruits are poor in the B vitamins in general and in minerals Certain fruits such as olives and nuts are remarkable and useful for their high lipid content

Vegetables particularly the leafy vegetables are second only to *fruits* as sources of ascorbic acid and are superior to fruits as sources of the B vitamins, provitamin A and minerals The importance of leafy vegetables in general, can however be overemphasized With a few exceptions they are very deficient in protein and the calorie content of leafy vegetables is

rice is even more important than wheat since the vast populations of the Orient subsist to a large extent upon that grain Other important grains are maize, oats, barley rye, and millet

The wheat kernel consists of three parts (1) an outer coat of *bran*, which comprises 12 to 15 per cent of the whole, (2) the *germ*, which is the actual embryo plant and forms 2 to 3 per cent of the whole, and (3) the *endosperm*, which forms the remaining major portion of the kernel and represents the initial food supply of the young plant In the preparation of white flour, the bran and germ are discarded in the milling process, so that the flour is essentially finely ground endosperm Such flour is poor in protein as compared with meat, both quantitatively and qualitatively In amount, protein comprises 10 per cent of wheat flour as compared with 20 per cent in meat or eggs In quality the protein of wheat is less well endowed with amino acid food factors The glutenin of wheat is complete, but gliadin is partially incomplete About 75 per cent of wheat flour is carbohydrate in the form of starch The lipid content is negligible

Wheat germ is ordinarily discarded in the milling process largely because its relatively high percentage of oil results in a tendency to become rancid and thus interferes with the keeping qualities of flour This is unfortunate since it is richer in protein minerals, and vitamins than is the endosperm Because of the fact that it forms such a small portion of the whole kernel, its contribution to the protein content is negligible, but in the case of the micronutrients it is most important It is particularly rich in the B complex Thus whole wheat flour which contains wheat germ is over four times as rich in thiamin as refined flour despite the fact that in terms of mass the germ contributes only some 3 per cent of the flour Ascorbic acid is absent in wheat as are vitamins A and D

Wheat, in common with most plant foods, is rich in minerals with the exception of sodium Plants, in general, are potassium rich and sodium poor as compared with animals so that man and herbivorous animals are forced to supplement their diet with sodium chloride Mineral content is most concentrated in the bran, which is discarded in the usual milling process Some two thirds of the carbohydrates of bran are indigestible pentosans so that bran is much used as "roughage" in the home treatment of constipation While undoubtedly often efficacious, the rough fibrous nature of bran makes it unfit for use in large quantities without medical supervision The irritating effect of bran may seriously aggravate disorders of the alimentary canal

Bread, while composed mostly of wheat flour—at least in the United States, possesses additives which affect its nutritive value, usually for the better Milk is frequently used in the preparation of bread, the nutritional value of this practice needing no comment The use of yeast in preparing

bread contributes to its vitamin content, as does the practice of manufacturing whole wheat bread in which germ and bran are included In recent years, enrichment with various vitamins has become common

Rice contains less protein (8 per cent as compared with 10 per cent) and more carbohydrate than wheat Rice is therefore somewhat less suited as a basic diet than is wheat The bran layers contain most of the thiamin content of rice, but again whole rice does not keep as well as does polished rice so that the economic pressures in favor of the latter are sometimes overwhelming Of historical interest is the fact that the first avitaminosis definitely characterized and cured was beriberi, found to occur among East Indians subsisting on a diet composed largely of white (polished) rice, and cured by the addition of brown (unpolished) rice

Maize differs from wheat in containing an appreciable (4 per cent) quantity of lipid material From this corn oil is derived It is an unsatisfactory food in itself since its proteins, while equal quantitatively to those of wheat, are far less complete It is also much poorer than wheat in the B vitamins so that among the lower economic strata of the southern part of the United States where the diet is composed in great part of cornbread and hominy grits pellagra has long been endemic

Fruits and Vegetables

Fruits, with the exception of the banana, contain no starch when fully ripened and most of the carbohydrate is in the form of utilizable sugars The mineral content is generally lower than in vegetables Fruits are characterized first by being among the richest natural sources of free monosaccharides (usually glucose or fructose), and of sucrose, which, together with their content of organic acids (usually malic acid or citric acid) and related compounds that contribute to that elusive element we call flavor, make them very pleasant eating Secondly, certain fruits represent our most valuable sources of ascorbic acid The juices of orange, lemon, grapefruit, pineapple, and tomato are recognized as excellent sources of that food factor Orange juice is for this reason commonly added to the infant diet at a very early age

Tomato juice is a rich source of provitamin A (see page 706), while pineapple and orange juices are fair sources Fruits are poor in the B vitamins in general and in minerals Certain fruits, such as olives and nuts, are remarkable and useful for their high lipid content

Vegetables, particularly the leafy vegetables are second only to fruits as sources of ascorbic acid and are superior to fruits as sources of the B vitamins, provitamin A, and minerals The importance of leafy vegetables, in general, can however be overemphasized With a few exceptions they are very deficient in protein, and the caloric content of leafy vegetables is

trifling Spinach for instance, although rich in iron is no richer than many other foods and considerably poorer than is meat It may contribute to loss of calcium from the body, *despite its high calcium content simply* because of its still higher content of oxalate ion which forms an insoluble and unutilizable salt with calcium The oxalate content of various leafy vegetables is a factor to be considered in the cases of those people with a tendency to form oxalate kidney stones (see page 669)

Starchy or mealy vegetables such as potatoes beans or peas differ in that they are richer in carbohydrate than most vegetables Potatoes are a poor but not entirely insignificant source of ascorbic acid In fact in parts of Europe where potatoes form a major portion of the diet and where citrus fruits are virtually unknown this vegetable alone stands between man and scurvy In potatoes as in other ascorbic acid containing vegetables the vitamin concentration is higher in the outer portions so that too enthusiastic peeling is nutritionally undesirable Beans and peas are unusually high in protein lima beans reaching a value of 7 5 per cent and soybeans nearly twice that concentration

REFERENCES

1 BOER J *et al* On the growth promoting factor for fats present in summer butter J Nutrition 33 339–358 1947

2 CALVIN M The path of carbon in photosynthesis VI J Chem Ed 26 639–657 1949

3 HANSEN A E AND BURR G O Essential fatty acids and human nutrition J A M A 132 855–859 1946

4 HICKMAN K C D AND HARRIS P L Tocopherol interrelationships Advances Enzymol 6 469–524 1946

5 HOLT L E JR *et al* Nitrogen balance in experimental tryptophane deficiency in man Proc Soc Exper Biol & Med 48 726–728 1941

dbook of

eds

0

rat

Lancet 2 491–492 1946

9 LOCKHART E E *et al* Study of the nutritional quality of dietaries by chemical analysis J Am Dietet A 20 742 746 1944

10 MACLEOD G AND SHERMAN H C *Recommended* dietary allowances in *A Handbook of Nutrition* 2nd edit pp 233 258 New York N Y Blakiston Co 1951

11 MANN G V AND STARE F J Nutritional needs in illness and disease in *A Handbook of Nutrition* 2nd edit pp 351 392 New York N Y Blakiston Co 1951

12 McCOY R H *et al* Feeding experiments with mixtures of highly purified amino acids VIII Isolation and identification of a new essential amino acid J Biol Chem 112 283 302 1935

13 NATH H et al Studies of the alleged growth promoting property of vaccenic acid J Nutrition 36 61 7"2 1948
14 NEWBURGH L H Obesity in JOLLIFFE N et al eds Clinical Nutrition pp 689-743 New York N Y Paul B Hoeber Inc 1950
15 RABINOWITSCH E I Photosynthesis Vol I New York N Y Interscience Publishers 1945
16 ROSE W C Amino acid requirements of man Federation Proc 8 546-552 1949
17 ROSEN F et al The effect of tryptophane on the synthesis of nicotinic acid in the rat J Biol Chem 163 343-344 1946
18 SOSKIN S AND LEVINE R Carbohydrate in nutrition in JOLLIFFE N et al eds Clinical Nutrition pp 208-235 New York N Y Paul B Hoeber Inc 1950
19 STIEGLITZ L J Nutrition problems of geriatric medicine in 4 M A Handbook of Nutrition 2nd ed pp 3 350 New York N Y Blakiston Co 1951
20 WARBURG O AND BURK D The maximum efficiency of photosynthesis Arch Biochem 25 410-443 1950
21 WILKINSON A B et al Nitrogen metabolism after surgical operations Lancet 1 533-537, 1950

CHAPTER 11

Digestion

It requires but little anatomical knowledge to realize that the body built around a hollow tube, the digestive tract Considered in its entire its functions are extremely simple It stores foodstuffs for a limited tin prepares foodstuffs for absorption, accomplishes their absorption, and r jects the unabsorbed remainder as feces Along with these primary fun tions, it acts to a very limited degree as an excretory channel and as location for the formation of certain vitamins by bacterial action

Most foodstuffs are not absorbable in nutritionally significant quantiti in the forms in which they are usually ingested The massive molecules proteins must be hydrolyzed to amino acids, or at least to small and simp peptides, before they can enter the body in significant amounts Amon the carbohydrates only the simple sugars or monosaccharides are absorl able Some difference of opinion exists among physiologists as to the degre to which unsplit fat can be absorbed It is, however, a fact that a ver effective enzymatic mechanism exists with apparently the sole function c hydrolyzing fat into its components, fatty acids and glycerol Clinical e perience has taught us that if this mechanism of fat hydrolysis is inact vated, the absorption of fats from the digestive tract is drastically d minished Digestion is a convenient term which includes all of the hydrolyti cleavages which occur in the gastrointestinal tract Digestion converts crud protein, carbohydrate, and fat into simpler components which can be effec tively absorbed

Our discussion of digestion and absorption will be limited to thos mechanisms which act to a sufficient degree to contribute significantly t the nutrition of the body From the point of view of nourishment, fo example, native animal and vegetable proteins are unabsorbable until they are completely or almost completely hydrolyzed On the other hand, minute amounts of animal or vegetable protein can certainly be absorbed through the mucous membranes of the mouth, of the rectum, and probably of most other portions of the alimentary canal Such absorption in minute amounts is amply demonstrated by the clinical fact that in a person who is hyper sensitive to a given protein, the ingestion of that protein or its introduction in an enema may, with striking promptness, induce the symptoms by which

his allergy is manifested These symptoms may be vasomotor rhinitis, bronchial spasm, urticaria, angioneurotic edema or even vasomotor syncope Such absorption also occurs in non allergic individuals This can be shown by the procedure known as local passive transfer One spot on the skin of the normal subject is sensitized by the endermal injection of a small amount of the allergic patient's serum That single spot on the normal subject can be caused to itch and redden by giving him the allergenic protein by mouth or enema However, the amounts of specific protein required to bring about these manifestations are remarkably small We are concerned here only with those mechanisms involved in the transfer of large amounts of food stuff from the digestive tract to the body proper

Our discussion will deal almost exclusively with enzymes and their activators We shall as might be expected emphasize the chemical aspects of digestion There are, of course also mechanical aspects the consideration of which belongs to the domain of physiology

THE SALIVARY GLANDS AND THEIR SECRETION

The salivary glands differ from each other somewhat in the type of secretion produced The parotid glands produce a thin saliva rich in starch splitting enzyme and containing relatively little mucin The sublingual and submandibular glands produce a thick secretion highly mucinous and with little enzymatic action Little information is available concerning the secretion of the buccal glands on account of the difficulty of isolating their secretion We shall concentrate our attention chiefly upon the parotid gland since it has been studied most intensively It is a compound tubular type of gland where secretory cells cluster around minute branches of collecting ducts When we examine stained sections with the microscope we find fine basal granulations in the cells These have been identified as pentosenucleic acid We recognize that the most significant product of these cells is a protein with enzymatic, in this case starch splitting activity We have here therefore an example of the association of cytoplasmic pentosenucleic acid with protein synthesis

One should recall that the innervation of the salivary glands is both sympathetic and parasympathetic In the case of the parotid gland, the parasympathetic stimuli reach the gland from the auriculotemporal branch of the trigeminal nerve with some fibers from the glossopharyngeal Use of electrical stimulation or of a parasympathomimetic drug like pilocarpine increases the secretory rate and the volume A moderate vasodilatation can be observed Sympathetic innervation is from the upper cervical chain passing through the superior ganglion This has no stimulatory action upon the parotid, from the other salivary glands sympathetic stimulation yields a scanty saliva of increased viscosity In the parotid, vasoconstriction and

contraction of the myoepithelial or basket cells can be observed, the basket cells lie immediately peripheral to the secreting acinar cells These two actions lead to an actual decrease in the size of the gland

Although the methods used may be criticized to some extent as being unphysiological, Lashley (24) observed basal secretory rates in normal adults of from 0 5 to 8 0 ml per hour per single gland He used a simple metallic collection disc held over the aperture of Stenson's duct by moderate suction The disc contained a compartment for collection and a compartment for storage which latter was attached to a measuring device Salivary secretion whether basal or reflexly stimulated has been shown to require the oxidation of glucose (1) A comparison of the composition of blood entering and leaving the parotid gland in dogs showed that from 0 8 to 2 9 mgm glucose were utilized per gram of gland per hour This was under basal or unstimulated conditions The mean value was 2 1 mgm This was compared with an independent measure of the oxygen consumption where the mean value was 1 2 ml per gland per hour which is the equivalent of 1 8 mgm glucose With stimulation an increase of 1 5 mgm glucose per gland per hour per ml saliva secreted was observed

The secretion of the parotid and other salivary glands contains in somewhat diminished amounts all the diffusible substances present in the blood In addition to these components, which need not be discussed in detail, we find characteristically in the parotid secretion the enzyme, *salivary amylase*, or according to the older terminology, *ptyalin* Among the salivary glands the parotid is the chief if not the exclusive producer of this enzyme Amylase is present in the saliva of newborn human infants and at all subsequent ages It has been crystallized (30) Its Q_{10} value in the physiological range is from 2 0 to 2 3, its temperature optimum, 40°C, its pH optimum, 6 6 It is inactive below pH 4 It loses 15 per cent of its activity on dialysis against dilute ammonia, the activity is restored by 0 01 M NaCl The action of the enzyme upon starch is a hydrolytic one in which successive units of maltose are split off, forming dextrins

A rather questionable component of saliva which may be characteristic

presence of the thiocyanate radical It must be admitted [illegible] highly non specific test and that attempts to confirm the presence of thiocyanate have not been uniformly successful It is demonstrable that the body can detoxicate sublethal doses of cyanide by oxidation to thiocyanate The presence of the supposed thiocyanate in saliva has therefore been explained as the end product of such a reaction involving cyanide groups ingested, inhaled with tobacco smoke, or produced by intestinal bacterial action There is really very little evidence for any of these hypotheses

Amylase is a characteristic secretion of the parotid glands A characteristic component of the secretion of the other sets of salivary glands is *mucin* This mucoprotein is most significant for its physical property of increasing the viscosity of the saliva and thereby increasing its lubricating effect Mucin gives the customary qualitative reactions of proteins, is not heat coagulable, can be precipitated with acid, and requires full saturation with ammonium sulfate for salting out The prosthetic group of mucin is mucoitin sulfuric acid or mucoitic acid which can be hydrolyzed to sulfuric, acetic, and glucuronic acids plus a hexosamine In the intact molecule it can be demonstrated that the acetic acid is combined with the hexosamine as acetylglucosamine Mucoitic acid is very similar to the chondroitic acid of cartilage which, however, contains acetylgalactosamine

It is impracticable to attempt an accurate measurement of the total 24 hour salivary output A reasonable estimate is about a liter and a half per day Since the salivary glands themselves weigh only 65 grams, this indicates a rather remarkable activity upon their part Mixed human saliva is of variable composition depending upon the relative contributions of the different sets of salivary glands It consists chiefly of water with up to 0 4 per cent protein (of which about 12 per cent is amylase) 0 2 per cent salts, and 0 1 per cent organic matter other than protein The specific gravity ranges between 1 002 and 1 008 Its viscosity is from 18 to 35 times that of water, and the pH, as determined in our laboratory on 45 healthy young adults, was 7 58 with a standard deviation of 0 36 pH units In this series the pH was measured promptly but without special precautions against loss of carbon dioxide Schmidt Nielsen (38) reported that with special precautions against loss of carbon dioxide the pH of fresh resting parotid saliva varied between 5 45 and 6 06 with an average of 5 81 For mandibular saliva the figures were 6 02 to 7 14, average 6 39 Various stimuli gave higher pH values Loss of carbon dioxide resulted in a rapid rise in pH to a limiting value of about 7 9

Salivary buffer capacity. Dreizen, *et al* (11) found that the buffer capacity of the saliva was higher in caries resistant as compared with caries susceptible individuals Their highest buffer capacities were found in malnourished patients relatively free from dental caries

The buffer capacity of a solution is defined by

$$db/d(pH)$$

where d(pH) is the increase of pH resulting from the addition of db of base, the addition of acid, which decreases the pH, is equivalent to a negative amount of base The slope of the pH neutralization curve is the reciprocal of the buffer capacity

Dreizen *et al* measured buffer capacity as the ml of 0 1 N lactic acid

required to lower the pH of the saliva sample from 7 0 to 6 0—this measures what might be called the physiological buffer capacity, since it is measured at a pH value comparable to that in the normal mouth

In our series, buffer capacity was measured both ways, for purposes of comparison those figures obtained by the "physiological" method seem more useful Of our 44 cases the mean value was 0 594 ml of 0 1 N lactic acid, with a standard deviation of 0 181 Dreizen *et al* found an average value of 0 615 ml in their group of nutritionally deficient, caries free cases, 0 484 ml in their well nourished patients with average caries activity, and 0 353 in their well nourished group with rampant caries

There can be no doubt that the physical and chemical properties of the saliva and the nature of its bacterial flora have some bearing upon the problem of dental decay Caries does not occur in unerupted teeth no matter how hereditary nutritional or endocrine factors may alter the susceptibility of the teeth to decay, teeth do not decay until they are exposed to the saliva The initiating factor may, therefore, be chemical or bacteriological, or a combination of the two Miller long ago proposed that the carious state was the result of a chemico parasitic process, the action of acids produced by the fermentation of carbohydrates in the mouth

Nord (35), summarizing the researches in this field since the time of Miller, states that caries is produced by the activities of micro-organisms which reach the amelo-dentinal junction through a defect in the enamel and then proceed to grow along the line The production of acid or hydrolytic enzymes or both by micro-organisms may result in a shrinkage of the dentine forming a space which will be filled by dental lymph This offers an excellent environment for microbial growth The enamel is attacked from the inside which is less resistant

Salivary calculus This term is applied to two separate and distinct forms of deposits originating from the saliva More commonly it designates the coating (tartar) of calcium and magnesium phosphates, with some admixture of carbonates and organic debris, which forms upon the teeth—natural or artificial—of many individuals The salivary origin of this deposit is indicated by its presence in greatest amount on the surfaces nearest the outlets of the salivary glands Alternatively, a salivary calculus may be a stone in a salivary gland or in its duct The presence of such stones, singly or in greater numbers, is known as *sialolithiasis* Stones occur most frequently in the submandibular gland or duct Their composition is similar to that of the salivary deposit upon dental surfaces

GASTRIC DIGESTION

The actual observation that gastric juice can dissolve meat was made and recorded by Spallanzani in 1783 Several famous patients, whose

direct fistulous openings from the stomach to the outside world attracted the scientific interest of their physicians have contributed painfully but effectively to our knowledge of gastric physiology and chemistry Let us call the roll Alexis St Martin whose opportunity to serve science came as the result of a gunshot wound was studied by Beaumont at the then isolated frontier post of Mackinac and was reported in 1833 (3) Beaumont s chief contributions aided by his not always co operative patient dealt chiefly with the inorganic composition of gastric juice It was from a specimen of gastric juice from Alexis St Martin that the definitive identification of HCl as the acid component was made Much later Fred V was studied by Carlson whose first report on this famous patient appeared in 1912 (8) The observations made by Carlson on Fred have been the backbone of our knowledge of gastric motility Moving ahead to 1943 we find Tom whose physicians Wolf and Wolff (46) filled in with great detail the relationships outlined by Cannon (7) Finally we have the unnamed woman aged twenty four who was studied by Crider and Walker at St Louis who demonstrated gastric responses to emotional stimuli opposite to those of Tom and her other male predecessors Anger and related emotions in the male subjects were with great consistency accompanied by unusual secretory activity of a gastric mucosa which became hyperemic with the onset of the emotional state Motility was increased In the anonymous St Louis woman similar emotional situations led to pallor of the mucosa with decreased gastric motility and secretion (9) The authors properly point out that if the observed differences are characteristic such differences in gastric response to emotion may explain the much greater incidence of peptic ulcer in men as compared with women

Gastric Acidity

An outstanding feature of the secretion of the stomach is its acidity Let us discuss this before considering the enzymatic functions we will show later that the activity of one of the significant enzymes is dependent upon the maintenance of acidity

Volume of gastric secretions A continuous spontaneous or basal secretion can be demonstrated in the majority of human stomachs Bengt Ihre (21) found the rate to vary from 15 to 117 ml per hour in 17 normal men and from 14 to 42 ml per hour in 6 normal women Subjects with high basal secretion were usually found to have high values for secretion under various stimuli which will be discussed later The basal secretion is not constant There are regularly recurring outbursts of secretory activity occurring during fasting about every 2 or 3 hours and lasting 10 to 30 minutes and accompanying phases of accelerated motor activity first observed by Carlson and called hunger contractions Wolf and Wolff found them

not to be associated with hunger sensations more than 50 per cent of the time

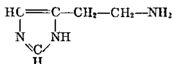
Neural stimulation of gastric secretion Although it is a familiar observation that the suggestion of food increases salivary secretion and intensifies the sensation of hunger it requires unusual experimental conditions to demonstrate the *concomitant increase in rate of gastric secretion* Passing a stomach tube in a normal subject is usually a distasteful process, and such a subject is resistant to suggestions by description or by odor which would, without the tube be appetizing Under hypnosis (21) it has been possible to demonstrate two to threefold increase in rate of secretion as a result of positive suggestion with accompanying increase of acidity comparable to that following a test meal Both hypnosis and normal sleep bring about a moderate increase in the basal secretion Sham feeding of experimental animals with gastric fistulae or pouches yields large outputs of gastric juice but such experiments give little information about physiological secretions in the human Numerous reports on human subjects particularly suited for this type of experiment by the presence of esophageal stricture and gastric fistula show that psychic secretion exists Secretion rates under such conditions range from 30 to 650 ml per hour

Neural stimuli reach the stomach by way of the vagus nerve Vagal stimulation direct or reflex increases volume acidity pepsin and mucin of the gastric secretion Complete vagotomy abolishes or decreases the acid secretion in about 75 per cent of human cases in which the operation has been performed for the relief of peptic ulcer (44) Levels of blood sugar below normal cause vagal stimulation of central origin with resulting increased volume and acidity of the gastric secretion Measurement and titration of gastric juice secreted after injection of 20 units of insulin which usually decreases the blood sugar to 0 050 per cent or less, tests the completeness of the operation Absence of secretory response to insulin indicates completeness of the operation Vagotomy or alternatively the use of anticholinergic drugs may be used therapeutically to depress excessive acid gastric secretion *Mechanical stimulation* of the gastric mucosa other than by distension does not induce acid secretion The output of mucus is increased

Chemical stimulation of gastric secretion Meat extracts broths and products of protein hydrolysis such as proteoses and peptones cause prompt and copious acid secretion when they are placed in the stomach This stimulation may be partly local by direct action upon the secreting cells But in animals with experimentally produced denervated gastric pouches (Heidenhain pouches) the introduction of such substances into the main stomach causes secretion in the pouch Since the only connection between main stomach and pouch is by way of the circulating blood the

stimulating substances must either themselves travel in the blood, or they must act to liberate a hormone from the mucosa of stomach or intestine. The stimulating substances do exert a mild secretagogue effect when injected intravenously, but this effect is not quantitatively comparable to the effect of their direct contact with gastric mucosa.

Edkins (13) made extracts of minced pyloric mucosa with peptone, glucose, or HCl. Injection of these extracts had strong stimulatory action upon acid secretion, compared with negative results with plain water extracts of pyloric mucosa. Presumably an active substance was formed by the interaction of the pyloric mucosa with peptone, glucose, or HCl. This active substance was designated *gastrin*. It was long confused with histamine (formula I), which is present in crude tissue extracts, and which will by itself produce a remarkable increase in the volume and acidity of gastric secretions. Histamine and gastrin are, however, separate substances with similar action. Extracts have been obtained from canine pyloric mucosa with gastrin activity. The effective dose of such extracts contains less than



I. Histamine

one sixth the threshold dose of histamine as measured by the cat blood pressure method (17). The effect of gastrin is solely upon gastric secretion. The mechanisms by which neural stimulation and hormonal stimulation of gastric acid secretion operate are distinct and independent. Vagal stimulation does not act by liberation of gastrin. This is demonstrated by the failure of vagally denervated gastric pouches in dogs to secrete acid when the main stomach is secreting acid under vagal stimulation (22). The structure and chemical composition of gastrin has not yet been established. Gastrin is usually designated as a hormone, although not all physiologists are agreed that it possesses all the requisites (19).

Caffeine prolongs the secretion of acid gastric juices, and usually increases the acid output. In doses of 500 mgm. caffeine has been used as a "test meal" in the clinical study of gastric secretion (33). *Alcohol* in dilute solution increases the volume and acidity of gastric juice whether given by mouth, by stomach tube, by rectal instillation, or intravenously. The direct contact of concentrated alcoholic preparations with the gastric mucosa tends more to cause mucus production. A suitable alcohol test meal for the stimulation of gastric secretion for analytical study consists of 50 ml. of 7 per cent alcohol, or 200 ml. of 5 per cent alcohol, both of these

are commonly used in clinical study Injection of 0 25 mgm of *histamine* produces in the average subject a prompt and maximal output of acid gastric juice The maximum is usually reached within half an hour If the hypothesis is accepted that histamine is a part of the normal mechanism for the stimulation of gastric secretion, it would be expected that antihistaminic drugs would depress acidity Actually no definite or significant effect of these drugs upon gastric acidity has been observed Parasympathomimetic drugs stimulate the output of both acid and enzymes *Atropine* blocks postganglionic cholinergic effects and has an action opposite to the parasympathomimetic drugs *Cholinesterase inhibitors* such as prostigmine permit increased duration of acetylcholine activity, with increase in gastric motility and secretion

Physiological inhibitors of gastric secretion Although the intravenous injection of glucose has only a minimal effect upon gastric secretory function the direct instillation of hypertonic *glucose* solutions into the duodenum definitely decreases the volume and acidity of the gastric juice (32) Intravenous administration of *amino-acid* mixtures has been observed to cause decreased gastric motility and secretion (9) The presence of *fat* in the small intestine is inhibitory to gastric secretion and motility The depressant effect of fat or sugar has been shown by Ivy and his co-workers to be the result of the release of an inhibiting substance or chalone to which they have given the name *enterogastrone* It has not been fully purified or identified, but can be separated from secretin (see page 455) and from cholecystokinin (see page 458) Another substance with a depressant action upon gastric secretion and motility has been isolated from urine and called *urogastrone* It acts in a manner very similar to enterogastrone but is not identical with it

Emotional disturbances may inhibit or excite gastric secretion Wolf and Wolff (46) report that in their male subject diminution of motility, circulation and acid production was associated with mental reactions of avoidance of or withdrawal from an emotionally loaded situation Stimulation of gastric function occurred when the emotional response was one of fighting back More frequent occurrence of the avoidance reaction was observed by Crider and Walker (9) in their female patient

Formation of hydrochloric acid Different varieties of cells compose the mucous glands in the stomach Of these cell types two have definitely established functions the body chief cells secrete pepsinogen, the parietal cells secrete hydrochloric acid Pure parietal secretion is a slightly hypertonic solution of pure hydrochloric acid at a concentration of about 0 17 N and a corresponding pH of 0 87 It is impossible to isolate the pure secretion of normally functioning parietal cells The figures just quoted are the result of indirect evidence which will be outlined briefly

The subcutaneous or intramuscular injection of histamine has been found to stimulate the acid secretion of the parietal cells with minimal action on the other cells composing the gastric glands Linde *et al* (26) tied off the stomachs of anesthetized cats and placed therein measured amounts of glycine solution which was later removed completely The volume increment was measured also the comparative titration values against standard alkali, and from these figures was calculated the HCl concentration of the parietal secretion A value of 0 17 N was the minimum figure A source of error leading to higher figures would be the possibility of absorption of the glycine solution

This minimal figure was in agreement with the work of Hollander (20) Instead of tying off the stomach in his experimental dogs he constructed Pavlov pouches A Pavlov pouch is a portion of the secretory mucosa of the stomach surgically rearranged to communicate directly with the outside of the body but retaining its original innervation from the vagus nerve Distinction should be made between this and the Heidenhain pouch mentioned previously, which is surgically entirely separated from the main portion of the stomach and deprived of all innervation vagus or otherwise

The two graphs (fig 16) represent experimental values obtained from histamine stimulated dogs with Pavlov pouches Both graphs indicate that with increasing acidity chlorides other than HCl tend to diminish Both lines intersect the acidity axis at a value of about 0 17 N, which appears to confirm this value of the concentration of HCl in pure parietal secretion

Since there is strong evidence that pure parietal secretion contains water, hydrochloric acid, and nothing else, it follows according to Hollander that somewhere in the parietal cell there must be a membrane which is permeable to water, hydrogen ion, halide ions, and to nothing else Figure 17 is an idealized picture of a single parietal cell Hollander cheerfully admits that the intracellular canaliculi have been rejected as artifacts by some histologists If such canaliculi really exist their walls would seem to be a reasonable location for the logically demanded membrane of such strictly limited permeability If we accept this idea that the canalicular membrane is permeable only to water, hydrogen ions and halide ions then any metabolic process which results in the extrusion of water by the cell would result in water secretion through the canaliculus containing those ions, hydrogen and halide which can pass that membrane Escape of these ions would leave an excess of sodium ions and hydroxide ions, which together with all other solutes present would be extruded through the less selective portion of the cell in equilibrium with extracellular fluid In summation then, Hollander's theory is that of a membrane hydrolysis of sodium chloride The slightly hypertonic concentration of hydrochloric acid probably represents a slight hypertonicity of the intracellular fluid as compared

with extracellular fluid Hollander postulates no osmotic work performed in the process just described Work must be done, however, by the cell in excreting water It had been previously demonstrated by others that there is increased glucose consumption and oxygen utilization during the process of active secretion of gastric hydrochloric acid

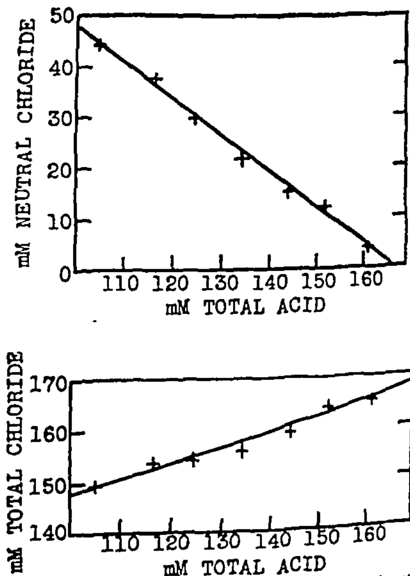


Fig 16 Experimental demonstration of maximum of gastric acidity in dogs at a concentration of 170 meq acid per liter (Hollander)

Some further light has been shed on the process going on within the parietal cell by the work of Patterson and Stetten (36) They devised an ingenious apparatus for the continuous measurement of the pH difference across the stomach wall They worked with isolated rat stomach and found that continuous oxygenation was necessary to maintain acid production

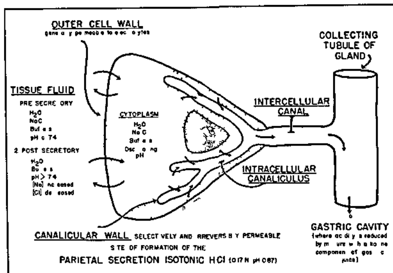
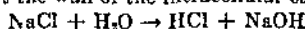
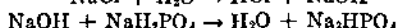


FIG 17 Schematic representation of the process of HCl formation in the parietal cell of the stomach (Courtesy of Dr Hollander)

The chemical reactions at the wall of the intracellular canaliculus



$$NaCl + H_2O \rightarrow HCl + NaOH$$



$$NaOH + NaH_2PO_4 \rightarrow H_2O + Na_2HPO_4$$

(and similarly for other buffer salts in the cytoplasm and lymph)

The energy factors

Chemical work—in the cytoplasm and lymph

Electrical work at the canalicular wall

Osmotic work—at the canalicular wall (zero)

Mechanical work—from the intracellular canaliculus to the open end of the gland tubule

Acid formation was inhibited by cyanide, fluoride arsenite and iodoacetate Tetramethyl *p* phenylene diamine which is an inhibitor of DPN and TPN linked enzyme systems had an inhibiting effect at low concentrations Others have shown that carbonic anhydrase and the niacin-containing DPN and TPN are abundantly present in parietal cells Animals with niacin deficiency show decreased acid secretion There is increased bicarbonate in the venous drainage of the stomach during periods of active acid secretion A difference in electrical potential can be demonstrated across the

gastric wall in which the mucosal side is negative to the serosal side in an external circuit These observations indicate that there is an orientation of the parietal cells which Patterson and Stetten consider as resulting from a stratification of enzyme systems inside the cell Figure 18 indicates their concept of this stratification By this mechanism a local high concentration of hydrogen ions may be developed at an area close to the exit from the cell on the gastric side Hydrogen ions are produced by the oxidation of carbohydrate intermediates utilizing a pyridine nucleotide coenzyme The

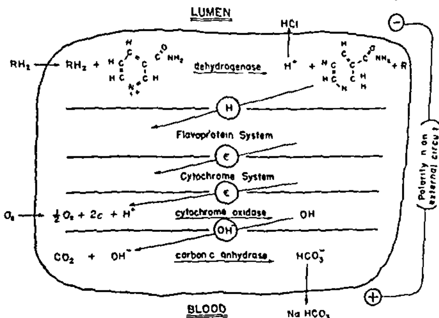


FIG 18 Stratification of enzymes in the parietal cell, according to Patterson and Stetten From Transactions of the First Conference on Metabolic Relations, Josiah Macy, Jr Foundation February, 1949

carbohydrate intermediate is represented by RH_2 in the diagram Note that two hydrogens are liberated from the carbohydrate intermediate, one of them appearing as hydrogen ion, the other being passed on to the flavo protein system which serves as a reductant of the neighboring cytochromes An electron passed on from cytochrome to cytochrome oxidase can catalyze the formation of hydroxyl ion from molecular oxygen If the system were not stratified, the hydroxyl ion would be immediately neutralized by the hydrogen ion formed in the first step Actually, the hydroxyl ions react with CO_2 in the presence of carbonic anhydrase This forms bicarbonate ion which diffuses into the blood stream as sodium bicarbonate An "alkaline tide" can be observed in the urine after meals while hydrochloric acid

is being secreted by the stomach An absence of alkaline tide indicates failure to secrete hydrochloric acid Patterson and Stetten have been unable to add histological proof to their concept of the stratification of these enzymes With this single exception all other points in this theory correspond to experimentally observed facts In summary the main points of their theory are

1 That the hydrogen ions arise from the reduction of pyridine nucleotide

2 That in the parietal cell the enzymes of the oxidation reduction systems are arranged in successive strata

Gastric Mucin

In its general composition the mucin of the gastric juice is similar to the mucin of saliva (see page 441) The secretion of mucin is increased by vagal stimulation and by local stimulation of the gastric mucosa, either mechanically or by irritant chemicals The buffer action of gastric mucin is responsible for only a small part of the difference in acidity between pure parietal secretion and mixed gastric secretion The difference depends rather upon inorganic buffers which are found in the native secretion and which can be removed by dialysis

The gastric juice contains both dissolved and undissolved mucus the latter being visible as strings or shreds The dissolved mucus may be precipitated from gastric juice with acetone Both the dissolved and the undissolved mucus consist of protein with polysaccharide prosthetic groups

Clinical Gastric Analysis

Specimens of gastric contents obtained by means of a stomach tube always contain a mixture of the secretions of the different glands of the gastric mucosa The concentration of HCl in such specimens never approaches the value of 0 17 N or 170 meq per liter which is characteristic of pure parietal cell secretion Furthermore contamination from saliva food residues and regurgitated duodenal contents almost invariably has occurred The pH may vary between 0 9 and 2 5 excluding those cases where hydrochloric acid is absent An occasional person apparently normal otherwise will show absence or minimal secretion of HCl This situation occurs more frequently in older age groups *Titratable acidity* of gastric contents is usually expressed in units or degrees of acidity defined as the number of ml of 0 1 N NaOH required to neutralize 100 ml of gastric contents By chance these old fashioned units are identical numerically with milliequivalents of acid per liter The latter more descriptive term should be used *Free acidity* is the result of titration with an indicator such as Topfer s reagent (dimethylaminoazobenzene red at pH 2 9 to

yellow at pH 4), which has a turning point at a low pH value Such a titration measures only strong acids and in gastric contents measures only the HCl which has not been neutralized or bound *Total acidity* is the result obtained with an indicator such as phenolphthalein (pK = 9 7) which includes not only free HCl but also acid salts organic acids and acid bound to protein

Fasting gastric juice usually contains less than 25 meq of free acid and less than 35 meq of total acid per liter The volume of contents usually obtainable from a normal fasting stomach is less than 50 ml Volumes of residual gastric contents greater than 120 ml and with free acidity over 50 meq per liter raise strong suspicion of peptic ulcer

The volume and acidity of the gastric contents increase following a test meal of low volume and low buffer capacity One hour after a simple test meal such as arrowroot cookies with 40 ml of water the gastric content of normal adults averages about 100 ml in volume with 40 meq of free acid and 55 meq of total acid per liter Variations among individuals are very great Similar values of free acidity can be obtained with a test meal of dilute alcohol (see page 445) Stimulation with histamine injections gives free acidities which are usually about 10 meq higher, but which occasionally are very much higher up to a limit of about 120 meq per liter When alcohol or histamine is the stimulus, there is usually no notable difference between free and total acidity If collections are made at short intervals after the test meal a curve of acidity against time can be plotted which is of some diagnostic value (6)

Achlorhydria is the inability of the gastric mucosal glands to produce any hydrochloric acid whatever Simple titration is not adequate to demonstrate achlorhydria Roth and Bockus (37) have emphasized that an adequate dosage of histamine of tested potency must be used with concomitant production of objective histamine effects, such as headache, flush or slightly lowered blood pressure Salivary contamination of the gastric collections must be rigidly excluded Samples should be taken every 15 minutes for 2 hours, and should be checked for decrease of pH as well as for titratable acid Stimulation with caffeine followed by histamine is suggested A patient should not be declared achlorhydric until several attempts to secure an acid response under the most favorable conditions have failed The demonstration of achlorhydria under these conditions excludes the diagnosis of peptic ulcer

An adequate dosage of histamine for eliciting acid gastric response, if this is possible contains 0 1 mgm of histamine base per 10 kilograms of body weight Histamine base is not injected as such but as a salt, 0 166 mgm of histamine dihydrochloride or 0 275 mgm of histamine diphosphate contains 0 1 mgm of histamine base Achlorhydria is a typical finding in

pernicious anemia, hence the rigid demonstration or exclusion of achlorhydria becomes an important diagnostic aid Simple hypochlorhydria or subnormal acidity has no special diagnostic import It is true that acid production is impaired or destroyed in cancer of the stomach but such impairment is not an early sign and is of minor diagnostic value in comparison with x ray and gastroscopic studies

Hyperchlorhydria and *hypersecretion* are characteristic statistically of patients with the tendency to develop peptic ulcer Excessive vagal stimulation, possibly a result of emotional stresses, appears to explain these excesses of gastric activity Gastric analysis is of less value than x ray in *the diagnosis of peptic ulcer, since the acidity ranges of normal subjects and* of ulcer patients overlap widely

Gastric Enzymes

Pepsin, the characteristic gastric proteinase is the product of the action of hydrogen ion upon pepsinogen, which is secreted by the body chief cells of the gastric mucosal glands Both pepsinogen and pepsin have been crystallized, both are proteins but crystalline pepsin is not a single molecular species It has been shown to contain at least two proteolytic components with different solubilities Pepsinogen is converted autocatalytically into pepsin at pH 4 6 or in more acid solutions Pepsin catalyses proteolysis most effectively at pH 2 or thereabouts, with some slight variation according to the protein used as substrate Pepsin shows slight enzyme activity at pH 4, none at pH 5 At pH 6 it is irreversibly inactivated The acidity of gastric juice is adequate to initiate the process of protein hydrolysis, forming acid metaproteins Pepsin catalyses the further hydrolysis of these already denatured proteins to fragments of molecular weight less than 1000, designated as *proteoses* if they can be salted out by full saturation with ammonium sulfate and as *peptones* if they can not

Studies with synthetic substrates show that pepsin preferentially catalyses the hydrolytic cleavage of peptide linkages involving the amino group of tyrosine or phenylalanine Protamines which are deficient in tyrosine and phenylalanine, are not hydrolysed by peptic action

There is a substance in the urine with properties similar to pepsin This *uropepsin* is pepsinogen which has entered the circulating blood from actively secreting gastric gland cells, and has been excreted by the kidney The uropepsin activity of the urine is very slight compared to the pepsin activity of the gastric juice, but the two increase or decrease together in disease Patients with peptic ulcer often show increased uropepsin activity Uropepsin is increased following administration of ACTH (41) and disappears following total gastrectomy

In the course of the peptic proteolysis of *casein*, the chief protein of

milk, insoluble *calcium paracaseinate* is precipitated if adequate Ca^{++} is present This is the case if milk is ingested, since milk is rich in Ca^{++} This hydrolysis of casein to paracasein and peptone has often been attributed to a special enzyme rennin Such an enzyme has not been demonstrated in human gastric juice The clotting of milk which occurs upon its entry into the infant or adult stomach may be adequately explained by a combination of (a) the hydrolysis of casein in the presence of pepsin with production of soluble paracasein and the precipitation of insoluble calcium paracaseinate, and (b) the precipitation of isoelectric casein by the free HCl of the gastric juice *Rennet* is a milk clotting preparation obtained by extraction of the fourth stomach (*abomasum*) of nursing calves This preparation has been used since prehistoric times in the clotting of milk for the first step in the making of most varieties of cheese

Gastric lipase has little physiological significance It is more consistently demonstrable in the gastric secretions of infants and children than of adults It can be differentiated from regurgitated pancreatic lipase (which is often present in gastric contents) by a difference in pH optima The optimal pH for gastric lipase varies from pH 5 5 to pH 7 9 (39), with a shift of the optimum 1 5 to 2 0 units to the acid side in the presence of calcium salts

The *intrinsic factor* of gastric juice is a substance which augments the action of orally administered vitamin B_{12} in promoting the maturation of red cells An additional proteolytic enzyme, designated as a cathepsin accompanies pepsin in the gastric secretion Its pH optimum has been measured at 4 7, which makes its digestive significance small in normally acid stomachs The gastric mucosa contains considerable quantities of the enzyme *urease* which catalyzes the hydrolysis of urea into ammonium carbonate The presence of a high blood level of urea diminishes the acid output in response to histamine stimulation (15) It has been postulated that urease is involved in the neutralization of gastric acid, particularly within cells where acid has entered by diffusion and that the activity of urease may in part explain the perennial question of why the pepsin of the stomach does not ordinarily bring about the digestion of the gastric lining

DIGESTIVE PROCESSES IN THE INTESTINE

Located in the duodenum and in the upper part of the jejunum, the duodenal glands (Brunner's glands) secrete a mucinous fluid which has a titratable alkalinity of about 0 03 N The entry of acid gastric effluent into the intestine stimulates duodenal secretion apparently by a liberation of a hormone designated as *duocrinin* (19) In this manner a part of the acidity is neutralized Further neutralization is accomplished by the pancreatic

juice which is about 0 10 N in titratable alkalinity and to a less extent by the bile which in the human is about 0 01 N in titratable alkalinity and by the secretion from the intestinal glands (crypts of Lieberkuhn) which is of variable alkalinity In spite of the considerable volume of alkaline secretions which enters the intestine the intestinal contents do not become more than slightly basic in reaction The average pH in the duodenum is 5 7 in fasting subjects and slightly more acid averaging 4 7 after meals In the lower portion of the ileum the fasting pH is about 7 2 which is comparable to the pH of the fe es on a normal mixed diet

The external or digestive secretion of the pancreas is absent or at best intermittent in the fasting state Vagal stimulation has little effect upon the volume output but increases the concentration of enzymes in the secretion The stimulation of the copious production of alkaline pancreatic juice is supplied by the hormone *secretin* which is liberated by the contact of acid with the mucosa of the duodenum Secretin can be obtained for experimental purposes by acid extraction of intestinal preferably duodenal

tration Secretin also increases the volume of bile secreted by the liver A second hormone *pancreozymin* can also be demonstrated in extracts of duodenal mucosa The action of pancreozymin is similar to the result of vagal stimulation causing increased output of pancreatic digestive enzymes with little alteration in volume or pH of the fluid The action of pancreozymin is not one of vagus stimulation since the action of the hormone is not abolished by atropine or other drugs which block cholinergic responses

Although the secreting cells of the pancreatic acini resemble in a general way the cells of the salivary glands the secretions are distinctly different The pancreatic secretion contains no mucin has a higher content of solids—

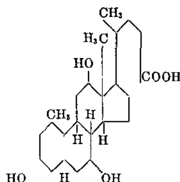
precursors which will be listed and described

Trypsinogen is a zymogen which is spontaneously but slowly converted to the active enzyme *trypsin* It is rapidly converted by *enterokinase* an enzyme of the intestinal secretion Trypsin is a proteinase or endopeptidase with an optimal pH between 8 and 9 The optimal pH for the conversion of trypsinogen to trypsin in the presence of enterokinase is between 5 and 6 The peptide linkages selectively hydrolysed in the presence of trypsin are those involving amino acids with basic groups Certain proteins which are resistant to pepsin such as protamines and histones are hydrolysed

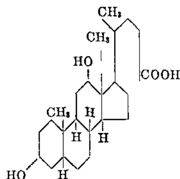
in the presence of trypsin. Trypsin acts slowly upon undenatured collagen, albumin, globulin or hemoglobin but acts rapidly upon the products of peptic digestion. *Chymotrypsinogen* is converted to active chymotrypsin by trypsin and acts upon peptide linkages involving amino acids with aromatic groups. The action of chymotrypsin is limited to products of peptic and tryptic digestion except that milk clotting comparable to that with pepsin occurs with chymotrypsin. The pH optimum is identical with that of trypsin. Both trypsin and chymotrypsin have been crystallized. The end products of physiological tryptic and chymotryptic hydrolysis are peptides of lower molecular weight than the proteoses and peptones which are produced by peptic digestion. There is some liberation of free amino acids, particularly those with aromatic groups. The *carboxypeptidase* of the pancreatic juice is a preformed enzyme which catalyzes the hydrolysis of end peptide linkages with free carboxyl groups. Such hydrolysis liberates free amino acids. To summarize digestion of proteins by the enzymes of pancreatic juice, native proteins are slowly hydrolysed and the products of peptic digestion are rapidly hydrolysed to peptides of molecular weight less than 1000, with some liberation of free amino acids. Simultaneously with the hydrolysis of protein, nucleic acids liberated by protein hydrolysis are hydrolysed to oligonucleotides with the aid of *polynucleotidase*.

Pancreatic amylase or *amylopsin* has an optimal pH for activity between 6.3 and 7.2, requires Cl^- for its normal activity, and yields maltose by the hydrolysis of starch or dextrins. Human pancreatic amylase has been crystallized from aqueous solutions at pH 6.4 to 6.6, yielding a product with activity identical with that of human salivary amylase (31). The amylase activity of the pancreatic juice of very young infants is so low as to be negligible, hence it is not advisable to feed starchy foods at early ages. The amylase values increase slowly, and do not reach adult levels until the age of about two years.

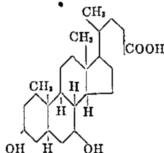
Pancreatic lipase, also called *steapsin*, is a preformed enzyme with optimal pH between 7 and 8. It promotes the hydrolysis of fats to fatty acids and glycerol. Although it is a true enzyme and not a zymogen, its activity is enhanced by bile salts and by Ca^{++}. This effect of bile salts is partly brought about by the emulsification of fats, which will be discussed in a later section. Since pancreatic lipase is already active, its escape from the pancreatic ducts as a result of injury to the pancreas is followed by hydrolysis of fat in the tissues with which it makes contact. Such hydrolysis of tissue fats is spoken of as *fat necrosis*. The proteolytic enzymes of the pancreas can also cause damage, since they are self activating, and their activation is catalyzed by contact with tissues. In addition to the ability to hydrolyze fats, pancreatic juice has enzymatic action on the hydrolysis or synthesis of cholesterol esters and of lecithin.



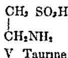
II Cholic acid (3,7,12 trihydroxycholanic acid)



III Desoxycholic acid (3,12 dihydroxycholanic acid)



IV Anthropodesoxycholic acid (3,7 dihydroxycholanic acid)



V Taurine

cent O_2, 7 2 per cent CH_4, 20 9 per cent H_2, and 59 0 per cent N_2 The concentration of H S ranged from zero to 0 0017 per cent Combustible gases H_2 and CH_4 may be present in explosive concentrations

BILE

No digestive enzymes are secreted by the liver into the bile The bile contains certain substances which in other ways contribute to the digestive process as well as some substances which are purely waste products Since the bile notably influences the composition of the intestinal contents it seems proper at this point to discuss its components and their origins The average daily human output of bile is estimated at 500 ml per 24 hours This output has never been measured in the human under strictly physiological conditions The human gall bladder contains approximately 50 ml of bile, which is there subjected to a process of concentration by the removal of water The normal gall bladder adds nothing to the bile except mucin (43) During the time that bile remains in the gall bladder the concentrations of most of its components increase from four to tenfold *Contraction of the gall bladder is stimulated by a hormone cholecystokinin,* which is liberated from upper intestinal mucosa by contact with fats, fatty acids, peptone, or dilute HCl

The *bile acids* are present partly as free acids and partly as salts The latter are designated as *bile salts* The total bile acid content, free acids plus salts, lies between one and two per cent in liver bile and may increase up to about ten per cent in gall bladder bile The chief bile acid in man is cholic acid (formula II) which makes up about 60 per cent of the total bile acid The remaining fractions are in approximately equal proportions desoxycholic acid (formula III) and anthropodesoxycholic acid (formula IV) These three bile acids of man occur to some extent as free acids or salts in the bile, but for the most part are conjugated in approximately equal amounts either with glycine or with taurine (formula V) Thus the *two bile salts which are actually most abundant* in human bile are those of the glycine and taurine conjugates of cholic acid, commonly called

[illegible]

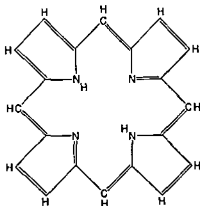
[illegible] onjugation with [illegible] is formed from cysteine or other S containing precursors Cholate can be measured in the blood (40), and has been found to range between 0 2 and 3 0 mgm per 100 ml, without great variation in individuals Values are increased in hepatitis and particularly in obstructive jaundice

The bile acids and bile salts are concerned *in the digestion and absorption* [illegible]

other digestive enzymes It is probable that some Ca^{++} is absorbed from the gall bladder, since normally only a sixfold concentration occurs there

Mucin is contributed chiefly by the gall bladder mucosa Liver bile contains practically no mucin but bile from the gall bladder contains up to 4 per cent When the cystic duct is obstructed the mucinous secretion accumulates in the gall bladder constituting the white bile of surgical terminology If the sphincter of Oddi is rendered non functional in experimental animals the gall bladder does not fill with bile and the mucinous secretion collects and may be analyzed (43) This secretion contains no cholesterol

Bile pigments are waste products derived from the porphyrin ring chiefly



VI Porphin

of hemoglobin Concentrations of bile pigment are observed in liver bile up to 0 07 per cent and in gall bladder bile up to one per cent Higher values have been reported post mortem (14) The bile pigments take no known part in digestion or absorption of foodstuffs They have considerable pathological significance however and warrant detailed consideration To understand their origin and behavior we must go back to blood pigment and its structure

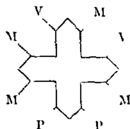
Porphyrins are derivatives of *porphin* according to organic chemical nomenclature Porphin (formula VI) is not a naturally occurring substance It has been prepared by degradation of naturally occurring porphyrins and by direct synthesis For comparison of the structures of porphyrins we can simplify our discussion by following Lemberg's convention and symbolizing porphin thus (formula VII), where the inward pointing angles signify nitrogens and the rest of the pyrrole rings are not represented The outermost angles are the methene bridges between the pyrrole rings

a matter of the lowering of the interfacial tension permitting stability of fine emulsions and partly the formation of soluble complexes Thus the bile acids and their salts aid in the emulsification of lipids prior to digestive hydrolysis Fine emulsions of lipids expose a greater surface to the action of the hydrolyzing enzymes and therefore digestion of such emulsions proceeds faster than the digestion of coarse suspensions After hydrolysis soluble complexes of bile acids with fatty acids form facilitating the absorption of fatty acids from the intestine Exclusion of bile from the intestine greatly decreases both the hydrolysis and absorption of fats About 90 per cent of the total bile acid output is returned to the body in the form of complexes with absorbed fatty acids constituting the *enterohepatic circulation* of bile acids The presence of bile salts is necessary for the effective absorption of carotene (see page 705) and the fat soluble vitamins

Lipids of the bile may occur in concentrations well over one per cent in healthy gall bladder bile The concentration in liver bile is variable and much less Phospholipids neutral fat and free fatty acids have been reported in highly variable amounts *Cholesterol* is not always the most abundant lipid in normal bile but its pathological interest has caused much study to be centered upon it It is present in normal liver bile in concentrations somewhat below those observed in blood Liver bile contains from 0 02 to 0 15 per cent cholesterol In normal gall bladder bile the concentration may reach 0 6 per cent In patients with cholesterosis of the gall bladder (deposits of cholesterol in the mucous membrane) or with cholesterol gallstones the concentration of cholesterol in the fluid portion of gall bladder bile may be 2 per cent or more Under these circumstances crystals of cholesterol may be detectable by microscopic examination of gall bladder bile obtainable from the intact patient by intubation of the duodenum followed by stimulation of gall bladder contraction by oleic acid or magnesium sulfate *Gallstones* have been observed only in man and in domesticated animals In the human patient gallstones are usually composed in whole or in part of cholesterol The disturbances leading to cholesterol stone formation are multiplex and beyond the scope of this text They include the possibilities of overproduction of cholesterol diminished solubility of cholesterol as a result of decreased acidity, bile acid content or fatty acid content of the bile and local alterations in the gall bladder as a result of infection A less common type of gallstone in man but more frequent in cattle and hogs is composed chiefly of calcium salts and bile pigment

Calcium is present in liver bile in a concentration equal to or slightly less than that in blood plasma 5 milliequivalents per liter Its presence in the intestine enhances the activity of pancreatic lipase and possibly of

protoporphyrin *IX*, but it is often called protoporphyrin *III* after the arrangement of the methyl groups only. Bile pigment can not be formed from free porphyrins (25). This judgment is based both upon physiological and organic chemical studies. Metal complexes of porphyrins can, however, be ruptured oxidatively at the alpha methene bridge by organic chemical procedures. In hemoglobin and other heme derivatives we are dealing with a metal complex of protoporphyrin *IX*. The metal is iron and the metal porphyrin complex is coupled to the protein globin. When sterile blood is allowed to stand, bile pigment and iron appear in increasing amounts (2). Conversion of blood pigment to bile pigment takes place in sterile blood at a rate too slow to account for the daily output. Since blockade of the reticulo-endothelial systems leads to diminished production of bile pigments in isolated spleen and liver, and since it is known from histological studies that cells of this system are phagocytic for red cells, the chief site



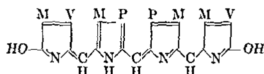
IX. Protoporphyrin *IX*

of bile pigment formation is considered to be within reticulo-endothelial cells, including the Kupffer cells of the liver.

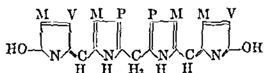
Biliverdin (formula X), which is protoporphyrin *IX* with the alpha methene bridge oxidatively ruptured, is the bile pigment first formed from the heme of hemoglobin. The ring breakage occurs while the porphyrin is still combined with iron and globin, and the bile pigment remains so combined, with the ring possibly still closed by a labile oxygen bridge. Such compounds of bile pigment with iron and protein are designated by Lemberg as verdohemochromes. This particular compound, where the protein is globin, is *verdohemoglobin*. The iron of verdoheme compounds is labile and is next split off, leaving biliverdin bound to the protein globin, forming *biliverdinglobin*. Biliverdinglobin acts as a hydrogen acceptor to lactic acid dehydrogenase and other dehydrogenase systems acting upon carbohydrate intermediates. By accepting two hydrogens biliverdinglobin becomes *bilirubinglobin*. Simple biliverdin added to blood is not reduced.

The measurement of bilirubin (formula XI) in the blood plasma or serum is usually carried out by some modification of the Hijmans van den Bergh reaction, which consists of adding a mixture of sulfanilic acid and sodium

nitrite to a solution of bilirubin. The bilirubin is thereby coupled with the diazotized sulfanilic acid, forming a much more deeply colored substance, *azobilirubin*. This reaction is weak or negative when applied directly to normal human serum—the *direct* Hijmans van den Bergh reaction. It is definitely positive when applied to normal human serum previously treated with alcohol—the *indirect* Hijmans van den Bergh reaction. This has been interpreted to mean that the bilirubin of normal serum is combined with globin as bilirubinglobin, and hence can not react with the diazo reagents, but that addition of alcohol splits the bilirubin from the protein and renders it reactive. This concept is by no means universally accepted. Both direct reacting and indirect reacting bilirubin have been crystallized (34) and show different properties. Digestion of serum with proteolytic enzymes does not change indirect reacting to direct reacting



X Biliverdin



XI Bilirubin

bilirubin. It may well be that the difference is one of actual structure of the pigment. Direct reacting bilirubin increases in blood serum in cases of obstructive jaundice or regurgitation jaundice, when bile pigment which has once passed through the liver cells is returned to the blood. The most common cause of such jaundice in a pure form is obstruction of the common bile duct, either by a gallstone or by pressure of a pancreatic tumor. Direct reacting serum can be converted to indirect reacting by mild oxidizing agents, such as very dilute hydrogen peroxide. The possibility, therefore, has not been excluded that the "indirect reacting bilirubin" of normal serum is actually a precursor of bilirubin, and that conversion to bilirubin occurs during the application of the analytical procedure. The

(29), show

man serum,

ive jaundice

or regurgitation jaundice the increased pigment in the serum is direct reacting bilirubin, in pure hemolytic jaundice the increase is in *indirect* reacting bilirubin.

Urobilins, which are fecal and urinary pigments, and *urobilinogens* which are colorless derivatives of bile pigments, result from further reduction of bilirubin in the intestine chiefly by the action of bacteria The vinyl side chains of bilirubin are reduced to ethyl, and one or more of the remaining methene bridges become methylene bridges The terminology of individual compounds in these groups is confusing For practical purposes it seems adequate to designate them simply as the colored urobilins and the colorless urobilinogens The inclusive fecal output of these substances is from 50 to 280 mgm per day A few milligrams of these substances are normally excreted in the urine, as a result of absorption of the substances from the intestine and their circulation in the blood The bulk of the absorbed urobilins and urobilinogens is not excreted in the urine, but is re-excreted in the bile Absence of urobilinogen or urobilin in the urine is therefore, in a jaundiced patient, evidence that the jaundice is obstructive in its origin, in other words that bile pigment is not entering the intestine This test may be misleading if there is obstructive jaundice with *E coli* infection above the obstruction in the bile ducts In such a case reduction of bilirubin will occur at the site of the infection and urobilins and urobilinogens will appear in the urine even though there is complete obstruction Also in very severe jaundice bilirubin may enter the intestine by diffusion in amounts adequate to form these reduction products The usual clinical test made on urine specimens is for urobilinogen, by adding a solution of p-dimethylaminobenzaldehyde in strong HCl

Bilirubin itself is not a normal urinary component It appears in the urine in jaundice, particularly in regurgitation jaundice It is commonly tested for by simple inspection, noting an abundant yellow or brown foam as opposed to the scanty white foam of normal urine Of the many chemical tests which have been proposed, the most reliable is Harrison's spot test, which utilizes strips of filter paper (Schleicher and Schull, No 470, is suitable) saturated with barium chloride solution and dried A drop of 25 per cent trichloracetic acid containing 0 9 per cent ferric chloride (Fouchet's reagent) on the surface line after the strip has been dipped into the urine for ten seconds gives a green color if bilirubin is present

The mechanisms just described for the formation of bile pigment from the heme of hemoglobin explain the production of almost 90 per cent of the normal output of bile pigment The origin of the remainder is not altogether clear Some may come from the breakdown of other heme compounds such as myoglobin, catalase and the cytochromes When isotopically tagged glycine is administered to a human subject, the incorporation of glycine into the heme of hemoglobin can be demonstrated, and also the formation of isotopically tagged bile pigment after the lapse of the normal life span of the red cells A portion of the isotope appears in

bile pigment however in the early days of the experiment indicating formation of bile pigment *other than from the heme of degraded erythrocytes* This early output of isotope in bile pigment is of greater proportion in pernicious anemia and still greater in congenital porphyria In a case of congenital porphyria (27) 31 per cent of the bile pigment excreted was demonstrated by the isotope technique to originate from sources other than the destruction of mature circulating red cells This suggests that the body utilizes glycine to form porphyrin derivatives which are not utilized in the production of the normal heme compounds and that these *are rather promptly converted to bile pigment and excreted* The fact that isotopically tagged protoporphyrin injected intravenously into the dog yields tagged bile pigment (28) makes this suggestion highly probable

INTESTINAL LYSOZYME

Lysozyme is an enzyme which depolymerizes certain amino polysaccharides which are obtained from definite species of bacteria for example *Micrococcus lysodeikticus* and *Sarcina lutea* Not only does this enzyme destroy by hydrolysis these specific mucopolysaccharides *in vitro* but it also attacks them as part of the parent organisms thereby lysing them *It can therefore be classified as an antibiotic (see Chapter 20)* In the human body we find lysozyme in the lachrymal secretion in the mucus secretion of the respiratory passages of the stomach and of both large and small intestine It is also present in human milk and in abnormal accumulations of body fluids such as the transudates which result from circulatory disturbances and the exudates which occur as the result of infections Lysozyme can be obtained in crystalline form using the white of egg as a starting material and lysozymes of plant and bacterial origin *are also known No substrate for lysozyme* has been found in the tissues of the human body although considerable investigation has been directed along these lines The lysozyme of tears has been shown to have a local damaging effect upon human colonic mucosa exposed through a colostomy opening (18) Increased amounts of lysozyme have been reported in the stool of patients with ulcerative disease of the intestine in the gastric ...se and in the mucus membrane of pylorus and duodenum of patients

The emotional state of the patient or subject seems to have so... upon the amount of lysozyme eliminated in the stools (18) Lysozyme does not hydrolyze the mucus of *gastric juice nor does it disturb* the production of mucus by surface epithelial cells (45)

ABSORPTION

Although the stomach is not anatomically adapted to the function of absorption moderate amounts of many simple substances enter the blood stream by way of the gastric mucosa Most foodstuffs are absorbed from the small intestine which is well adapted to this function by its great length and by its specialized epithelial lining Because of the villi the absorbing area is much larger than would be expected from the length and size of the small intestine Absorbed foodstuffs may leave the small intestine by one of two paths The first path is by way of the blood capillaries in the walls of the intestine and especially those in the villi thence to the mesenteric veins and the portal vein Any absorbed material which takes this path passes through the liver before it enters the general circulation This is important because the liver is able to adapt the concentration and the chemical structure of many of the absorbed materials for utilization in other tissues The second path of absorption is by way of the lymph vessels of the intestine the large lacteals and the thoracic duct The materials which take this path reach the blood more slowly but more directly because the thoracic duct empties into the venous system near the heart

Absorption of Inorganic Substances

Not enough water is removed by the small intestines to cause any notable decrease in the fluidity of the intestinal contents Thus the material which passes through the ileocecal valve has about the same consistency as the chyme which passes through the pylorus This is not because no water is absorbed in the small intestine but rather because copious secretions pour into the intestine and contain in them sufficient water to compensate for that which has been absorbed

There is every reason to think that water Na^+, and Cl^- are able to move freely in both directions, either in or out of the small intestine Sulfate ion also seems diffusible although it was formerly considered difficult to absorb The absorption of Ca^{++} is favored by the presence of vitamin D and by an acid pH, and is diminished by an alkaline pH and the presence of phosphate and carbonate

Absorption of Protein

The amino acids formed by digestive hydrolysis of protein foods are diffusible and sufficiently water-soluble to allow their prompt entry into both blood and lymph vessels of the intestinal villi The greater portions of the amino acids are carried by the blood vessels which fact may simply

reflect the more rapid rate of blood flow as compared with lymph flow The removal of amino acids from the intestinal contents is so effective that the limiting factor appears to be the *rate of amino acid liberation by* protein hydrolysis in the intestine

Proteoses and peptones are also water soluble and more or less diffusible *They are taken up by intestinal cells, within which further hydrolysis* occurs Analytically detectable increases of polypeptide concentration occur in the portal blood of experimental animals following protein meals The changes in amino acid concentration of systemic blood following protein absorption (see Chapter 14) are more uniform and predictable leading to the conclusion that the absorption of protein is chiefly in the form of amino acids

There is nevertheless evidence that molecules larger than amino acids may be absorbed For instance, strepogenin (47) which is a polypeptide passes into the blood Also the phenomena of food allergy suggests that foreign proteins may be absorbed at least in small amounts intact or relatively so because we know that after proteins have been hydrolyzed to constituent amino acids they are no longer capable of giving the specific reactions which one observes in allergy Thus it is hard to see how people who are sensitive to certain protein constituents of foods could react positively unless some of the proteins were absorbed and reached the sensitive tissue directly

Absorption of Carbohydrates

We have seen that carbohydrates are digested to monosaccharides *Sugars other than monosaccharides are not absorbed* from the intestine unless taken in relatively high concentration, and if so absorbed they are promptly eliminated by the kidneys For instance, if sucrose is eaten in *large amounts, it may be detected in the blood and in the urine* Normally however, the sugars which are absorbed are monosaccharides, and the pathway of absorption is through the portal circulation to the liver

These simple sugars are absorbed at *different rates* Cori has reported experiments on rats (10) in which sugars were given by stomach tube giving the following relative rates of absorption

D Galactose	110
D Glucose	100
D Fructose	43
D Mannose	19
L Xylose	15
L Arabinose	9

The much greater speed with which glucose and galactose are *taken up* suggests that there must be some special mechanism for their absorption

A plausible theory which has not yet been supported by completely adequate evidence is that the sugars are phosphorylated in the intestinal mucosal cells If glucose and galactose are so phosphorylated this would explain their rapid rate of absorption because they would promptly be removed from the cells and the back pressure against further absorption would be kept very low so that diffusion from the higher concentration in the intestine would continue unrestricted The idea that glucose and galactose may be phosphorylated is supported by the observation that monoiodoacetic acid and phlorizin which prevent phosphorylation also delay absorption Experimental hyperthyroidism increases the intestinal absorption of glucose galactose and oleic acid where it is supposed that phosphorylation occurs, but it does not increase the intestinal absorption of xylose alanine or calcium lactate where phosphorylation is not thought to be necessary This evidence is in line with the fact that in dogs resorption of glucose from the glomerular filtrate in the renal tubules of the kidney was increased by thyroxine and decreased by phlorizin However thyroxine did not increase the tubular resorption of galactose A deficiency of vitamin B complex produces a pronounced decrease of intestinal absorption in rats but does not influence the absorption of xylose It is known that the B complex supplies components to the phosphorylative enzyme system

The Absorption of Lipids

Free fatty acids may form a small part of the diet but for the most part they are combined as neutral fat or more complex lipids The salts of fatty acids, which are called soaps, are probably rarely present as they do not exist as such except at a pH higher than 9, and this degree of alkalinity does not occur in the gastrointestinal tract The mechanism of absorption of lipids has been hotly debated and there is still more than one point of view There is no question but that free fatty acids are liberated by the action of digestive lipases This hydrolysis is usually considered to be complete or nearly so with no difference between animal and vegetable fats Absorption of the fatty acids occurs exclusively in the small intestine aided by the bile acids The fatty acids are, with few exceptions water insoluble but can combine with bile acids to form water soluble complexes which can enter the surface of the absorbing cells of the intestine Presumably the water soluble complex is broken up within the cell membrane The bile acid portion is in part extruded back onto the intestinal surface of the cell and in part returned to the liver by way of the portal circulation The fatty acids are esterified to fats and phospholipids within the intestinal cell and removed chiefly (70 per cent or more) by way of the intestinal lymphatics and the thoracic duct *Glycerylphosphorylcholine* can be identified in the intestinal cells and can be presumed to

combine there with some of the fatty acids and pass them along in the form of lecithin, the remainder being *resynthesized to triglycerides* Further formation of phospholipid occurs in the liver (see page 528)

Frazer (16) has dissented from the concept of fat absorption just stated He postulates partial hydrolysis of fats (30 per cent or less) and the absorption of a complex of residual fatty acid monoglycerides and diglycerides with bile acids by way of the lymphatics, and direct transport of fatty acids by way of the portal vein to the liver

In an experiment which goes contrary to Frazer s partition hypothesis Berry and Ivy (4) cannulated the thoracic ducts of dogs and then introduced into the alimentary tract mineral oil in various emulsions which ranged in particle size from 200 to 0 5 microns and less They obtained no evidence that during a period of 15 to 24 hours there was absorption of the mineral oil in sufficient amounts to cause the lymph in the lacteals to become cloudy or milky or to cause a chemically detectable increase in the amounts of unsaponifiable material in the lymph or thoracic duct, or to cause any chemically detectable loss of mineral oil from the lumen of the intestine The importance of the lymphatic pathways for fat absorption has been further shown by Bloom *et al* (5), who introduced tripalmitin or palmitic acid labeled with C^{14} into rats Whether given as free acid or triglyceride,

duct, which

Other autho

neutral fat or fatty acids For instance Tidwell (42) found this to be true in the rat Absorbed fatty acid has been found to reappear as neutral fat in the lymphatics, already recombined with glycerol

Phospholipids lecithin in particular, may be *hydrolyzed by pancreatic* enzymes before absorption It has been demonstrated that phospholipids also may be resynthesized in the intestinal walls Cholesterol appears to be absorbed in two forms *in a soluble complex with bile salts, and* as cholesteryl esters formed with higher fatty acids These esters are soluble and are regularly found in the blood serum and have also been found in chyle

can occur in

to choles

red with

fat is absorbed by way of the lymphatics The absorption of cholesterol is not a critical process in human nutrition, since the body can synthesize its own cholesterol in adequate supply (see Chapter 13)

Sprue is a disease in which absorption of foodstuffs from the small intestine is notably diminished, with resulting nutritional failure, glossitis and macrocytic anemia The absorption of fatty acids, glycerol and sugars is

particularly affected The stools are bulky and contain excess fat (*steator rhea*) Diminished phosphorylation may be demonstrated but the cause of the disturbance has not been clearly defined and may be multiple (12) Vitamin B_2 is the most effective single therapeutic agent but effective treatment requires also a diet high in proteins and in all vitamins

REFERENCES

1 ANREP G V AND CANNAN R K The metabolism of the salivary glands II The blood sugar metabolism of the submaxillary gland J Physiol 56 248–258 1922

2 BARKAN G AND WALKER B S The red blood cell as a source of the iron and bilirubin of the blood plasma J Biol Chem 131 447–454 1939

3 BEAUMONT W *Experiments and Observations on the Gastric Juice and the Physiology of Digestion* Plattsburg New York F P Allen 1833

4 BERRY I M AND IVY A C Partition hypothesis for intestinal absorption of fat Am J Physiol 162 80–87 1950

5 BLOOM B *et al* The quantitative significance of the lymphatic pathway in transport of absorbed fatty acids J Biol Chem 184 1–8 1950

6 BOCKUS H L *Gastroenterology* Vol I *The Esophagus and Stomach* pp 198–230 Philadelphia and London W B Saunders Company 1943

7 CANNON W B The influence of emotional states on the alimentary canal Am J M Sc 137 480–487 1909

8 CARLSON A J Contributions to the physiology of the stomach I The character of the movements of the empty stomach in man Am J Physiol 31 151 168 1912

9 CRIDER R J AND WALKER S M Effect of intravenously administered amino acids on the stomach of a woman with a gastric fistula Arch Surg 57 10–17 1948

10 CORI C F The fate of sugar in the animal body I The rate of absorption of hexoses and pentoses from the intestinal tract J Biol Chem 66 691 715 1925

11 DREIZEN S *et al* Buffer capacity of saliva as measure of dental caries activity J Dent Research 25 213 222 1946

12 DURANT T M AND ZIBOLI I A Sprue A consideration of etiology differential diagnosis and management M Clin North America 33 16 1 1680 1949

13 EDKINS J S The chemical mechanism of gastric secretion J Physiol 34 133 144 1906

14 ELTON N W Bilirubin concentrations in the human gallbladder Am J Clin Path 6 81–90 1936

15 FITZGERALD O AND MURPHY P Studies on the physiological chemistry and clinical significance of urease and urea with special reference to the stomach Irish J M Sc 6th series No 292 97 159 1950

16 FRAZER A C The absorption of triglyceride fat from the intestine Physiol Rev 26 103 119 1946

17 FRIEDMAN M H F AND KING E N Presence of a specific gastric hormone (gastrin) in the dog s pyloric mucosa Federation Proc 6 107 194

18 GRACE W J *et al* Studies of the human colon I Variations in concentration

i ... with hi ... situation and emotional state Am J M Sc 217 211 ... 1949
1... ... M I Gas r ntestinal hormones Physiol Rev, 30 33-90 1950
2 H ... F The mposition and mechanism of formation of gastr c acid ... Science 110 57-63 1949
2 ... B. Human gastric secretion Acta med scandinav, Suppl 95 1938
22 J ... H D ... HOLLANDER F Critical evidence that vagal stimulation ... release g... Proc Soc Exper Biol & Med 76 49-50 1951
23 K ... L The quant ... and composition of human colonic flatus Gastroenter ... 12 782 ... 1949
24 L... K S Reflex secretion of the human parotid gland J Exper Psychol 1 461-493 1916
25 LEMBERG R, AND LE... E J W Hematin Compounds and Bile Pigments New York Interscience Publishers 1949
26 L... ..., et al Experiments on the primary acidity of the gastric juice Acta physiol scandinav 14 220-232 1947
27 LONDON I M et al Porphyrin formation and hemoglobin metabolism in con genital porphyria J Biol Chem 184 365-371, 1950
28 LONDON I M et al Conversion of protoporphyrin to bile pigment Federation Proc 10 217 1951
29 MALLOY H T AND EVELYN K A The determination of bilirubin with the photoelectric colorimeter J Biol Chem 119 481-490 1937
30 MEYER K H et al Amylolytic enzymes X Isolation and crystallization of alpha amylase from human saliva Helvet chim acta 31 2158-2164 1948
31 MEYER K H et al Purification and crystallization of human pancreatic amylase Arch Biochem 18 ... 1948
32 MUIR A Carbohydrate metabolism and gastric secretory activity Quart J Med 18 235-261 1949
33 MUSICK V H et al A simplified caffeine gastric test meal for the diagnosis of peptic ulcer J A M A 141 839-841 1949
34 NAJJAR V A Crystallization and prope... of "direct" and "indirect" biliru bin from human serum Federation P... ..., 1951
35 NORD ... The cause of dental c... ... Dent J, 81 309-316 1946
36 PATTER... W B AND STETTEN, DE... ...udy of gastri... Cl formation Science, 109 256-258, 1949
37 RATH J ... D BOCKUS, H L C ... tamine a... Gastro enterolog... 4 377, 1950
38 SCHMIDT ... The pH of par... lar physiol scandin... 110 1946
39 SCHO... ... D VOLQVARTZ ... ia... ...iol scand ... 1946
40 SHERLOCK ... WALSHE, V liver d... 223 234
41 SMITH, G ... peptic hormone ..., 1
42 TOWELL ... ... studies
43 WALKER ... gel Midd...

44 WALTERS W AND BELDING H H III Physiological effects of vagotomy J A M A 145 607-613 1951
45 WANG K J *et al* Action of lysozyme on gastrointestinal mucosa Arch Path, 49 298-306 1950
46 WOLF S AND WOLFF H G *Human Gastric Function* 2nd edit New York, N Y Oxford University Press, 1947
47 WOOLLEY D W Strepogenin activity of peptides of glutamic acid Federation Proc 6 424 1947

CHAPTER 12

Carbohydrates and Diabetes

Metabolism is a word with broad coverage It includes everything which happens to foodstuffs in the body, from the time and place of entrance to the time and place of exit In this broad sense, metabolism includes digestion, absorption, intermediary metabolism, and excretion The word is often used in a more restricted sense, covering only intermediary metabolism—those chemical changes which occur after absorption of a substance into the body and until the substance or its waste products are irrevocably committed to an excretory channel By tradition, and to aid understanding of this complex process it is customary to subdivide metabolism according to the type of raw material involved Following this useful custom, we shall deal in this chapter with carbohydrate metabolism and in later chapters with lipid, protein, water, mineral, and oxygen metabolism These subdivisions are helpful but not altogether rational We shall see that the metabolic pathways of different substances cross and recross, and that many significant interconversions are possible

Diabetes mellitus is the most important of the metabolic diseases It involves the fundamental processes of carbohydrate, fat, and protein metabolism with devastating effect upon human efficiency, longevity, and fertility It has rowelled investigators into intense and sustained activity, and at the same time guided them through the maze of the metabolic pattern by the very disturbances it produced In no other disease has the chemical approach been so rewarded in increase of understanding, in improvement of the patient's situation, and in the uncovering of new problems

The word *diabetes* derives from the Greek, meaning "a passing through" In classical Greek, diabetes means a siphon In English, diabetes means the chronic excretion of an excessive volume of urine Modified by an adjective meaning tasteless *diabetes insipidus* is a disease characterized by the excretion of large volumes of very dilute urine This uncommon disease involves the antidiuretic hormone of the posterior lobe of the pituitary, and will be discussed in Chapter 15

In ordinary medical terminology, when diabetes is mentioned, the adjective *mellitus*, meaning 'honeyed', is appended or, if no other modifier is used, is understood *Diabetes mellitus* is a disease characterized by the excretion of glucose in the urine and an increase in the concentration of glucose in the blood The designation of this disease as a diabetes is proper, since there is usually an increase in the volume of urine Diabetes mellitus has been recognized as a serious medical problem for more than two thousand years In the Sushruta Samhita, which has been ascribed to the 6th century B C (52), diabetes is recognizably described, and the medical student of that time was taught that

> Secretions or discharges should be tested with the organ of taste The sweet taste of the discharges should be inferred from the fact of their being or not being swarmed with hosts of ants or flies " (6)

Dobson in 1775 separated a sugar from diabetic urine by evaporation Chevreul did not isolate glucose from grapes until 1815 The two sugars were found identical

Diabetes is no rare disease There are more than a million diabetics in the United States, where diabetes stands ninth among the causes of death and is responsible for one death out of 40 Although there are significant differences in the onset and course of diabetes depending upon age, race, and nutritional status, it appears in all population groups, and is significant in all specialties and subspecialties of medical practice The pediatrician will see fewer cases than the general practitioner or the geriatrician, but those cases which occur at an early age present a more difficult problem in their individualized medical care

The objective signs of diabetes are glycosuria and hyperglycemia, usually accompanied by polyuria *Glycosuria* is the presence of glucose in the urine, demonstrable by any one of several simple chemical tests, of which Benedict's (see page 476) is most commonly used *Hyperglycemia* is elevation of the concentration of glucose in the blood above its normal level of 0 1 per cent to levels which in untreated diabetes usually lie between 0 2 and 0 5 per cent In less than 5 per cent of cases of diabetes does the blood sugar ever go over 1 0 per cent One case with a blood sugar slightly over 2 0 per cent has been reported (32) *Polyuria*, or an increase of urinary volume above the normal upper limit of 2 liters in 24 hours, is a direct result of the excretion of glucose in the urine, the glucose increases the effective osmotic concentration of the urine and opposes reabsorption of water in the renal tubules The relationship between glucose output and urine volume is not mathematically exact Patients may have glycosuria with normal urine volume

Less objective signs of diabetes are *polydipsia* and *polyphagia*—increased

intake of fluids and food The untreated diabetic is thirsty in response to the water depletion resulting from polyuria and is in a metabolic state showing many of the characteristics of starvation This metabolic state will be explained in this chapter, where the normal metabolism of carbohydrate will be considered in comparison with its waste in the diabetic

MONOSACCHARIDES

Only three of the simple sugars or monosaccharides are of dietary significance These are glucose, fructose, and galactose Carbohydrate foods are utilizable in metabolism only to the degree that they are convertible by hydrolysis into these simple sugars Glucose and fructose occur free in fruits and honey and in food products obtained by the hydrolysis of higher carbohydrates Galactose never occurs free in natural foodstuffs *Mannose* could be of significance except that it is present in foodstuffs only in the carbohydrate portion of serum and egg proteins (56) It is absorbable and can be converted into glycogen Since it is quantitatively insignificant in human nutrition, we shall give it no further consideration

Within the body other monosaccharides may be identified, usually as components of more complex molecules Two trioses, *glyceric aldehyde* and *dihydroxyacetone*, occur as phosphoric esters They are intermediates in the utilization of the dietary sugars, and will be discussed in that connection

The two types of pentoses involved in the structure of nucleic acids (see Chapter 7) are synthesized in the body Dietary pentoses are not utilized in their formation Another pentose, L-*ketoxylose*, appears in the urine of patients with the rare metabolic disease, *essential pentosuria* This pentose is formed in the body from glucuronic acid (17)

Analytical Reactions of Simple Sugars

All monosaccharides reduce Cu^{++} to Cu^{+} in solution, over a wide range of pH *Benedict's qualitative solution* is widely used for the detection of sugar in urine It is prepared by dissolving 173 grams of sodium citrate, and 100 grams of anhydrous sodium carbonate in 800 ml of hot water To this is added slowly 17 3 grams of crystalline copper sulfate dissolved in 100 ml of water The volume is finally made up to 1 liter at 20°C with water Five ml of Benedict's solution is placed in a test tube and pre-heated to boiling Five-tenths ml of urine is added with mixing The mixture is kept boiling for one minute or held in a boiling water bath for five minutes, then allowed to cool slowly to room temperature The presence of 0 05 per cent of hexose or pentose in the urine will be indicated by a yellow precipitate of Cu_2O Before the precipitate settles, it will appear as a greenish turbidity Higher concentrations of sugar are indicated by a more copious precipitate appearing more quickly and redder in color This alkaline copper

solution is reduced by all hexoses and pentoses under the conditions specified and also by *maltose* which does not occur in urine, and by *lactose*, which may be present in the urine of lactating women. L-ketoxylose, fructose and high concentrations of glucose will reduce Benedict's solution even at *room temperature* if allowed to stand overnight.

Benedict's solution may be prepared in a modification suitable for quantitative estimations of the sugars which reduce Benedict's qualitative solution. The quantitative modification contains per liter

	grams
Copper sulfate crystalline	18.0
Sodium carbonate anhydrous	100.0
Sodium citrate	200.0
Potassium thiocyanate	125.0

Use the same steps in preparation as for the qualitative solution, adding the copper sulfate last. This solution is not suitable for qualitative testing. When it is heated with a reducing sugar the Cu^+ formed is precipitated as white CuSCN. To titrate the sugar in urine exactly 25 ml. of Benedict's quantitative solution is pipetted into a porcelain dish of about 100 ml. capacity. *Solid sodium carbonate is added in an amount sufficient to saturate the solution at the boiling temperature.* The solution is kept boiling and the urine is added slowly from a burette at a rate just enough to replace the loss of volume of the boiling solution. Stirring is necessary to prevent caking of the reactants in the dish. The end point is the disappearance of blue or green color from the system. In titration of simple sugar solutions the end point is distinctly seen in a nearly colorless mixture. When urines *are titrated brown pigments usually obscure the end point and make the determination less exact. The calculation of the concentration of reducing* sugar is based upon the fact that 25 ml. of Benedict's quantitative solution is completely reduced by 50 mgm. of glucose, 52 mgm. of fructose, 54 mgm. of galactose, 67 mgm. of lactose, or 74 mgm. of maltose.

There are many other tests for sugar which involve reduction of Cu^{++}. In organic chemistry *Fehling's solutions* are used for detection of aldehydes and ketones. Benedict's and other reagents involving Cu^{++} reduction are modifications of Fehling's test for more specific purposes. The Benedict reagent is less sensitive than Fehling's to certain normal urinary components such as uric acid and creatinine, hence is more specific for sugars in the urine. Reduction of Bi^{++} to metallic bismuth is the basis of another set of sugar reagents (e.g. Nylander's solution) little used in America but often mentioned in European publications. Ag^+ is reduced similarly to metallic silver. The deposition of a silver mirror on a glass surface by the reduction of ammoniacal silver salts with glucose is an amusing demonstration but less reliable than Cu^{++} reduction as a clinical test.

Reduction of Cu^{++} in acid solutions is much more rapid with monosaccharides than with the reducing disaccharides If time and temperature are controlled, acid Cu^{++} solutions such as *Barfoed's* can be used to differentiate lactose and maltose from the monosaccharides

The principle of Cu^{++} reduction can also be applied to the measurement of the blood sugar which is chiefly glucose The classical American method of blood sugar estimation is that of Folin and Wu (21) It involves the preparation of a clear protein free, fat free blood filtrate by the addition to diluted blood of equivalent amounts of sodium tungstate and sulfuric acid Blood proteins are precipitated by the liberated tungstic acid, blood lipids are adsorbed to the protein precipitate which is separated by filtration A measured volume of blood filtrate is mixed with an alkaline cupric tartrate solution, heated for a definite time and then reacted with a solution of phosphomolybdic acid In acid solution reduction of phosphomolybdic acid to a blue complex molybdate by Cu_2O occurs The amount of Cu_2O depends upon the amount of reducing sugar originally present in the blood filtrate Colorimetric comparison is made against a standard glucose solution simultaneously treated exactly like the blood filtrate This is one of the simplest of blood sugar methods It has the disadvantage of being distinctly non specific The values obtained are higher than the true sugar concentrations of the blood since the alkaline cupric tartrate is reduced by other substances in the blood than reducing sugars By this method the normal range of fasting blood sugar is 0 08 to 0 12 per cent The true glucose value as measured by more specific but less simple methods is 0 06 to 0 08 per cent

Reduction of substances other than copper may be applied to quantitative blood sugar measurements Ferricyanide may be reduced by glucose to ferrocyanide and the concentration measured colorimetrically as prussian blue This reaction is the basis of a micro-method (20) whereby sugar can be measured in 0 05-ml samples of blood which can be obtained by skin puncture For estimations of urinary sugar reduction of organic compounds is commonly used for example the reduction of picric acid (5) and of dinitrosalicylic acid (62)

Most sugars undergo partial decomposition at temperatures below their melting points This leads to inexactness in melting point determinations However, the identification of sugars by melting point can be done accurately by first preparing a derivative of the sugar with phenylhydrazine or a substituted phenylhydrazine The derivatives which may be phenylosazones or phenylhydrazones, have sharp melting points and may be readily identified after recrystallization It is a common practice to attempt identification by examining crystalline derivatives under the microscope This is reliable in skilled hands and under favorable conditions but is full of pit

falls for the occasional analyst The proper choice of reagents for the identification of a particular sugar is discussed in specialized monographs

A number of color reactions have been devised for sugars and their derivatives which depend upon the liberation of furfural or furfural derivatives when carbohydrates are decomposed with strong acid. All carbohydrates give the *Molisch reaction* a few drops of 5 per cent alcoholic solution of alpha naphthol or thymol are added to the solution in question, which is then stratified over concentrated sulfuric acid in the presence of carbohydrate a violet color develops at the plane of contact *Seliwanoff's reagent* is 0 05 per cent resorcinol in 12 per cent aqueous HCl solution. It is specific for ketohexoses under the conditions specified Fructose being the only important ketohexose in metabolism it is used as a qualitative test for that sugar To one volume of solution in question is added 5 volumes of the reagent and the mixture is heated in boiling water The appearance within a few minutes of a red color, a condensation product of resorcinol with hydroxymethylfurfural is a positive result The *orcinol reaction* for pentoses is carried out in a strongly acid solution, and depends upon furfural formation The simple procedure described in many laboratory manuals is unreliable Drury (14) has listed the proper precautions to be taken in pentose studies with orcinol

The dicarboxylic acids produced by oxidation of simple sugars by nitric acid are all soluble except the mucic acid produced from galactose. This test is used to identify galactose free or in combination as in lactose.

More specific than most color or reduction tests are the microbiological procedures for identification of sugars Most strains of ordinary baker's or brewers yeast *Saccharomyces cerevisiae* will bring about the fermentation of glucose or fructose to ethyl alcohol and CO_2, but yield negative results with galactose It is possible, however to adapt a strain of yeast so that it will ferment galactose In all microbiological identifications it is highly important to run controls with known sugars, since sudden changes in the metabolic capabilities of a pure strain may occur, to say nothing of the possibility of contamination of the culture with extraneous organisms. Many other organisms may be used in sugar identification. Conversely, the ability to metabolize certain carbohydrates and not others is a common taxonomic criterion used by microbiologists

The rotation of the plane of polarization of plane-polarized light by sugar solutions offers another measurement which can be utilized for analytical purposes

DISACCHARIDES

In addition to the three simple sugars of dietary importance there are also three significant double sugars lactose, maltose and sucrose

occurs in the milks of all milk yielding species and only in milks and milk products It is a condensation of glucose and galactose is hydrolysed by a specific digestive enzyme lactase and is absorbed as the two simple sugars The occurrence of *maltose* in untreated foodstuffs is insignificant It is a product of the hydrolysis of starch inside or outside the body It has a specific enzyme maltase which during digestion promotes its hydrolysis to two molecules of glucose *Sucrose* is the characteristic double sugar of plants and foods derived from plants It is the common sugar of the table and the kitchen the product of sugar cane sugar beet and sugar maple It occurs in smaller amounts in many other edible plants Its hydrolysis to the absorbable monosaccharides glucose and fructose is aided by the digestive enzyme *sucrase* sometimes called *invertase*

Analytical Reactions of Disaccharides

Lactose and maltose reduce alkaline Cu^{++} solutions they reduce acid Cu^{++} solutions more slowly than monosaccharides hence can be distinguished by the use of Barfoed s reagent Sucrose does not reduce unless hydrolysed to monosaccharides Maltose and lactose form specific phenyl osazones sucrose does not but slowly forms glucosazone as a result of hydrolysis Lactose yields a positive mucic acid test sucrose a positive Schwanow reaction Sucrose causes an alkaline solution of diazouracil to turn green (45) Maltose and sucrose are fermented by *Saccharomyces cerevisiae* lactose is not

POLYSACCHARIDES

Glycogen is the animal polysaccharide which is composed of glucose units and which hydrolyses in acid solutions to glucose It is soluble in and stable to alkali which permits its separation from other tissue components which hydrolyse in alkaline solutions It is present in all metabolically active animal cells in yeast and many other micro organisms but not commonly in plant cells Estimates of its molecular weight range from $2\ 7 \times 10^6$ to $3\ 5 \times 10^6$ isolated preparations are highly polydisperse (45) It forms a violet red or brown substance when treated with iodine It is insoluble in alcohol and can be purified by alternate solution in alkali and reprecipitation with alcohol For analysis of tissues for glycogen it is separated in this manner then converted by acid hydrolysis to glucose which is estimated by one of the methods described for simple sugars In the later discussion of carbohydrate metabolism we shall be particularly concerned with the glycogen of liver and of muscle

Serum polysaccharide is a term used to designate the carbohydrate fraction of the blood serum proteins This fraction makes up from 1 5 to 3 per cent of the serum proteins The carbohydrate content of the alpha and

beta globulins is the highest, but all protein fractions of blood serum contain carbohydrate The hydrolysis products of serum polysaccharide are glucosamine, galactose and mannose Evidence from animals with acute liver poisoning from phosphorus or benzene indicates that the liver is the site of formation of serum polysaccharide (66) In the human, following blood or plasma loss, or in any situation where there is an increased demand for protein formation increased levels of serum polysaccharide have been observed

Starch is the usual storage form of carbohydrate in plants It is composed of glucose units, linked by the type of glucosidic linkage seen in maltose On acid alkaline, or enzymatic hydrolysis it yields smaller polysaccharide fragments known as dextrins, together with maltose Continued acid or alkaline hydrolysis, or enzymatic hydrolysis with maltase yields ultimately glucose Iodine converts starch into a blue substance which is reported to be an oxidized compound containing no iodine (65) The function of starch in human physiology is purely nutritional it is hydrolysed by digestive enzymes to glucose and absorbed as such

Inulin is a less common plant polysaccharide composed of fructose units It is of no nutritive value to vertebrates all of which lack enzymes to promote its digestive hydrolysis It can be utilized by certain microorganisms and is of value to microbiologists in identification and to physiologists for renal clearance tests in man (see Chapter 18)

Cellulose, a glucose polysaccharide with cellobiose linkages, is the chief structural component of plants It is not digested in the human, although herbivorous animals can utilize its glucose liberated by bacterial action Considerable quantities of cellulose are consumed in the average human diet, with only the dubious advantage of added bulk to the fecal output In this same class of undigestible carbohydrates fall numerous *hemicelluloses*, *pentosans* and *pectins* (uronic acid polysaccharides) These substances, particularly the pectins, have applications in gastrointestinal therapy but they take no part in human carbohydrate metabolism

THE ANABOLISM OF CARBOHYDRATE

The completion of the digestive process leaves all nutrient carbohydrates in the form of glucose fructose, and galactose Absorption transfers these simple sugars from the lumen of the intestine to the blood of the intestinal capillaries The venous blood of the intestine passes by way of the portal vein to the liver The simple sugars in the liver face three immediate alternatives —escape through the liver into the hepatic vein and the general circulation storage in the liver as liver glycogen, and catabolism in the liver The last will be considered in a later section along with catabolism of carbohydrate in the body as a whole

Escape through the liver is demonstrated by the increase in circulating blood sugar following ingestion of a test dose of sugar, or following an *ordinary meal* The circulating blood sugar is chiefly glucose Its concentration is approximately 0 1 per cent, more exactly 0 06 to 0 08 per cent in the fasting subject Most mammalian species show a similar blood sugar level Glucose and other simple sugars are distributed equally in the water of plasma and the water of blood cells Since the cells contain less water than plasma, the concentration of sugar in plasma is greater than in cells or in whole blood Following the ingestion of a test dose of more than 100 grams of glucose, the *average person will show an elevation of blood* sugar above the "renal threshold" of 0 180 per cent, and glucose will be excreted in the urine The increase of blood sugar following an ordinary meal is seldom more than 0 04 per cent, which does not induce glycosuria There is, however, a normal output of reducing sugars in the urine, *less than* 0 9 gram per 24 hours, not enough to cause reduction of *Benedict's solution*

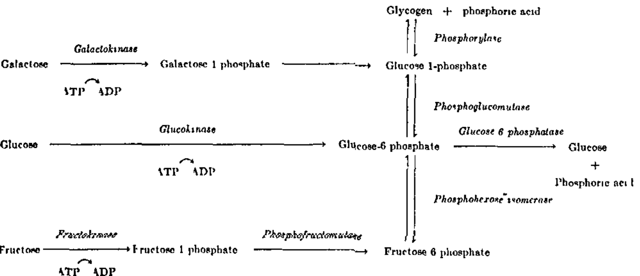
There is a measurable difference in the sugar level of arterial and venous blood This is called the A V difference and depends upon (a) the current rate of utilization of glucose by the organ supplied, and (b) the rate of blood flow through that organ

Storage in the liver is in the form of glycogen The liver can store glycogen up to 6 per cent of its weight, or a total of a little over 100 grams Liver *glycogen can be formed from* all *three of the simple* nutrient sugars—glucose, fructose, and galactose, the process is called *glycogenesis* (table 29) The first step in glycogen formation from glucose is the transfer of a phosphate group from adenosine triphosphate (ATP) to the sugar, forming glucose 6 phosphate (Robison ester (formula I)) The ATP is thereby converted to adenosine diphosphate (ADP) Fructose *is also phosphorylated* in the liver, with the formation of fructose 1 phosphate Enzymes known as *glucokinase* and *fructokinase*, or collectively as *hexokinase*, catalyze these reactions, which take place within the cells of the liver In the fermentation of sugars by yeast a single hexokinase is effective for glucose fructose, and mannose Simple sugars diffuse freely in *and out of cells* phosphate esters of sugars do not Thus the hexokinase reaction permits the capture of glucose by cells The hexokinase reaction is not reversed since it involves the transfer of phosphate from a high energy *to a* low energy bond

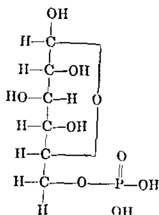
Fructose 1 phosphate is in equilibrium with *fructose 6 phosphate*, the conversion being catalyzed by an *enzyme* designated as *phosphofructomutase* Fructose 6 phosphate (Neuberg ester (formula II)) is reversibly convertible to glucose 6 phosphate, catalyzed by *phosphohexose isomerase* Glucose 6 phosphate and glucose 1 phosphate (Cori ester (formula III)) are interconvertible under the influence of *phosphoglucomutase*

TABLE 29

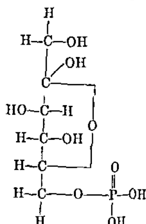
Glycogenesis and glycogenolysis in liver



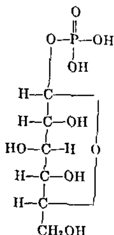
Glucose 1 phosphate is converted to glycogen and inorganic phosphate reversibly, catalyzed by *phosphorylase* (11) The equilibrium under physiological conditions is at about 77 per cent glycogen to 23 per cent glucose 1



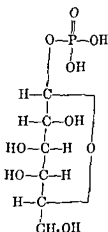
I Glucopyranose 6 phosphate
(Robison ester)



II Fructofuranose 6 phosphate
(Neuberg ester)



III Glucose 1 phosphate
(Cori ester)



IV Galactose 1 phosphate
(Kosterlitz ester)

phosphate Such an equilibrium indicates no great change in free energy No high-energy phosphate bond is required to re convert glycogen to glucose 1 phosphate The easy interconversion of glycogen and glucose 1 phosphate is quite different from the hydrolysis of glycogen or starch by amylase No detectable polysaccharide formation occurs when glucose or

maltose is exposed to the action of amylase. Glycogen is at the same stage of utilization, energically speaking, as the hexose phosphates with which it is in equilibrium, and one step ahead of glucose which must be esterified by expenditure of a high energy phosphate bond before it can be metabolized in the usual manner by glycolysis. As might also be judged from the even equilibrium, glycogen is in a state of dynamic interchange. This can be demonstrated by tracer studies which indicate the liver glycogen has a half life of about a day.

Galactose is not phosphorylated by ATP in the presence of yeast hexokinase. A galactokinase (64) has been identified in a lactose fermenting yeast which catalyzes the formation of galactose 1 phosphate (Kosterlitz ester (formula IV)) from galactose and ATP. Note that galactose 6 phosphate is not formed; this ester has never been found in biological systems. Galactose 1 phosphate has been isolated from the livers of galactose fed animals; this ester can be converted to glucose 1 phosphate in liver.

The rate of glycogenesis is one of the significant factors in determining the outcome of *sugar tolerance tests*. Such tests consist of the administration to a fasting subject of a known amount of a pure sugar, followed by repeated measurements of that sugar in the blood or the urine or both. The *glucose tolerance test* is of particular significance in the diagnosis of diabetes, where glycogenesis is inhibited by mechanisms to be described later. If 50 to 100 grams of glucose is given by mouth to a normal subject with fasting blood sugar of about 0.10 per cent, the venous blood sugar will rise within the next hour to about 0.15 per cent and will have started its return to normal before the end of the hour without having induced glycosuria. By the end of the third hour the blood sugar will have returned to normal, probably to a little below the original fasting level as a result of the stimulus of the ingested sugar to glycogenesis. The same dose of glucose given to an untreated diabetic whose fasting blood sugar would be above the normal value already, induces a rise of blood sugar increasing for about two hours and not returning to the original level by the end of the third hour. Normal subjects who have been on a low carbohydrate diet may show a diabetic type of tolerance curve.

The *galactose tolerance test* involves the administration of a test dose of 40 grams of galactose. Following such a dose, the normal person eliminates less than three grams of galactose in the urine during the 5-hour period following the test dose. In liver disease involving defective glycogenesis more galactose is excreted. The shape and timing of the blood galactose curve in the normal is comparable to that of the glucose tolerance test, increasing in height and duration when larger doses of galactose are given. With the 40 gram dose, blood galactose levels may go as high as 0.08 per

cent in the normal With an 80 gram dose, blood galactose may go slightly above 0 10 *per cent* *The galactose tolerance test* is in common clinical use as a measure of the glycogen function of the liver

Experimental hepatectomy in animals is followed by a sudden fall in blood *sugar, since the stored liver glycogen is removed and hepatic gluco*neogenesis (see page 488) is stopped It is somewhat surprising how little effect liver disease in man has in altering blood sugar, except in terminal stages The *reserve capacity of the liver is so great that, except under* stress, little change in carbohydrate metabolism is noted Blood sugar changes in response to fasting, to insulin, and to sugar administration are likely to be exaggerated and *protracted in liver disease* Lowest blood sugar levels occur before breakfast, following the longest fasting period The anabolism of galactose is specifically a liver function—hence the use of galactose tolerance tests

Glycogen of liver cells can break down again to glucose, but not to fructose or galactose The transformation of glycogen to glucose occurs only in liver cells, not in cells of other tissues or organs This breakdown is called *glycogenolysis*, and takes place in three steps First, glycogen reacts with phosphate ion to yield glucose 1 phosphate This is a true phosphorolysis, and is the reversal of the last step in glycogenesis As in that step, the enzyme is phosphorylase The second step changes glucose 1-phosphate to glucose 6 phosphate This is another reversal, catalyzed by phosphoglucomutase The third step involves a new enzyme, a phosphatase specific for glucose 6 phosphate and found only in liver cells, which converts glucose 6 phosphate irreversibly to glucose Unlike glycogen and the glucose phosphates, glucose can leave the liver cell and contribute to the blood sugar *In vitro*, potassium ions have been shown to promote glycogenesis, and sodium ions to promote glycogenolysis (28) in rabbit liver slices The storage of glycogen in the human binds 0 36 mEq potassium for each gram of glycogen This potassium is released with glycogenolysis

The ability of the liver to store ingested carbohydrate as glycogen and later to release it as glucose was first noted a century ago by *the French* physiologist Claude Bernard The question *for which he experimentally* sought the answer was, "What is the source of the sugar in diabetic urine?" In those years there was no suggestion that the pancreas was at fault Claude Bernard studied normal *animals, trying to determine which organs* contributed glucose to the blood This he found that the liver could do In animals not absorbing sugars from the intestine, there was a higher *level of glucose in the blood of the* hepatic vein than in that of either the

[illegible] when he found

tudy led

This was

the first of many discoveries in normal biochemistry inspired by a primary interest in diabetes

Glycogen in Muscle

The mechanism for the production of glycogen from glucose in muscle is identical with that in the liver The first two steps in its breakdown are also the same, forming first glucose 1 phosphate and then glucose 6 phosphate, the necessary enzymes phosphorylase and phosphoglucomutase, are at hand in muscle No conversion of glucose 6 phosphate to glucose can occur, however, since the enzyme glucose 6 phosphatase does not occur in muscle Glucose 6 phosphate can not itself leave the muscle cell by diffusion, its only possible alternatives are glycolysis (see page 491) or resynthesis to glycogen by reversal of the steps mentioned above

The formation of glycogen from fructose is more direct in muscle than in liver Muscle fructokinase forms fructose 6 phosphate directly, omitting the intermediate formation of fructose 1 phosphate (see table 29)

Effects of Hormones Upon Glycogen Storage

Adrenalin promotes the breakdown of glycogen to glucose-6 phosphate In the liver, and with adequate liver glycogen, this results in increased liberation of glucose to the blood Muscle glycogen is converted to glucose-6 phosphate which, for lack of the specific phosphatase can not be converted to glucose The glucose 6 phosphate undergoes anaerobic glycolysis (see page 491), forming lactic acid which can escape from the cell Transported by the blood to the liver, the lactic acid is converted to glycogen Thus in the well fed animal adrenalin decreases liver glycogen, and in the fasting animal may increase it

On the basis of experimental evidence summarized by Cori (11), he has proposed an integrated concept of the actions of three hormones on hexokinase the *anterior pituitary* produces a specific inhibitor of liver and muscle hexokinase, the *adrenal corticoids* intensify and protract the pituitary inhibition of hexokinase, *insulin* releases hexokinase from inhibition, favoring thereby the formation of glucose-6-phosphate from glucose, and thus indirectly favoring the formation of glycogen Subsequent studies have for the most part been consistent with this triple concept (3), although there has been some evidence which is directly contradictory (57) This mechanism for the endocrine control of hexokinase, or more specifically of glucokinase, will be introduced repeatedly into the remainder of this discussion of carbohydrate metabolism as a good working hypothesis and not as a set of facts fully proven

Glycogen storage disease is a very rare metabolic error, inherited as a Mendelian recessive, in which the liver is greatly enlarged and its cells

are gorged with glycogen The glycogen content of the granular leukocytes is *increased* which is a valuable point in diagnosis The blood sugar is low and there is likely to be ketonuria (see page 525) Homogenates of liver taken at autopsy from patients with glycogen storage disease will not hydrolyze glycogen under conditions which permit its hydrolysis by homogenates of normal liver In another form of glycogen storage disease the glycogen accumulation occurs predominantly in cardiac and skeletal muscle

Gluconeogenesis

Other substances than hexoses and glycogen can form glucose in the body, and glucose so formed behaves no differently from glucose derived directly from the carbohydrates of the diet The formation of glucose from precursors which are not hexoses or hexosans is called gluconeogenesis and is almost exclusively a function of the liver Gluconeogenesis has however been observed in the kidneys of hepatectomized animals Glucose can be formed from lactic acid, pyruvic acid, and all the intermediates between glucose or glycogen and lactic acid in the glycolytic mechanism (see table 30) Glucose can also be formed from the acids of the citric acid cycle (see page 496) from mannitol and sorbitol, from glycerol (with triose phosphate as an intermediate) and from the amino acids listed as glycogenic on page 534 Gluconeogenesis from these amino acids can occur only after the amino group has been removed by oxidative deamination or by transamination (see Chapter 14) Gluconeogenesis from *fatty acids* does not occur, meaning that glycogen or glucose can not be increased in the whole animal at the expense of fatty acids Tagged carbon atoms of administered fatty acids may appear in glucose or glycogen, but there is no net gain in glucose or glycogen since an equal number of carbon atoms is oxidized to CO_2 This problem of the interconversion of fatty acid and carbohydrate will be taken up in more detail in the next chapter The common intermediate in gluconeogenesis is glucose 6 phosphate It will be recalled that this ester can not cross cell boundaries, and must be used or transformed in the cell where it is formed

The glycocorticoids of the adrenal have been shown to promote gluconeogenesis from amino acids Recalling the previously mentioned adrenocortical augmentation of the inhibitory effect of the pituitary upon hexokinase—the first enzyme involved in glucose utilization—we can see that the combined effect of the glycocorticoids is to retard utilization and speed formation of glucose, in short to maintain the level of blood glucose Gluconeogenesis is also stimulated by thyroxin, and by lack of insulin In the normal person gluconeogenesis prevents hypoglycemia during prolonged fasting

PRODUCTION OF PHYSIOLOGICAL SUGARS

Fructose is the characteristic sugar of the seminal plasma Its origin from blood glucose has been demonstrated by Mann and Parsons (40) who found in diabetic rabbits levels of fructose in semen proportional to the glucose of the blood Normal human semen contains highly variable amounts of fructose, up to 0 64 per cent or 32 mgm per ejaculate The values are higher in human diabetics Fructose disappears from the seminal fluid following castration or hypophysectomy and reappears with administration of testosterone or gonadotrophin respectively

Lactose has already been designated exclusively as the sugar of milks Its precursors in the guinea pig are glucose and glycogen (39) Since either of these substances will serve as a substrate for lactose production, it is probable that the mechanism is one of phosphorolytic glycogenolysis No parallelism between blood sugar and milk sugar comparable to that described for seminal fructose has ever been reported The insulin requirement of diabetic women is often decreased during lactation while in normal lactating women blood sugar tends to be low

Pentoses Mammals can not phosphorylate dietary pentoses and therefore cannot metabolize them The pentoses of nucleic acids are built in the body, probably from hexoses Rabbit kidney can convert glucose or fructose, plain or phosphorylated, into pentose (47) The exact structure of the pentose so formed has not yet been established An enzymatic mechanism has been demonstrated in liver, brain, and kidney (13) by which D ribose 5 phosphate appears as a result of the oxidation and decarboxylation of hexose phosphates The phosphorylated pentose is a step in a pathway of sugar utilization alternative with anaerobic glycolysis (see page 491), as well as a highly probable mechanism for the production of pentose

Uronic acids, which are carbohydrate derivatives enter into the structures of a number of substances important in physiology *Glucuronic acid* is the best known member of this group, and is a component of hyaluronic acid chondroitin and mucoitin sulfuric acids, and heparin It has never been detected in the free form in nature (1) nor has the mechanism of its production been explained although its formation from glucose has been demonstrated by tracer methods (42) Glucuronic acid conjugates with many phenols and alcohols, either extraneous or metabolic origin, to form glucuronides which are excreted in the urine Conjugation with glucuronic acid is quantitatively the most significant mechanism of so-called *detoxication*, which is a term used to indicate alterations by oxidation, reduction, or conjugation of foreign or waste products within the body prior to their renal excretion In many instances such alterations actually do decrease the toxicity of the substance or increase its rate of elimination

An enzyme, *beta-glucuronidase*, catalyzes the synthesis and hydrolysis of numerous glucuronides including those of substances of extraneous origin such as menthol and borneol and those of physiological origin such as estriol and pregnanediol (19) High activity of this enzyme is observed in liver spleen, lung, endocrine tissues, and leukocytes Some degree of activity is observed in all fresh tissues Uterine beta glucuronidase activity is subnormal in ovariectomized mice, but is increased by injections of natural or synthetic estrogens The activity is greatly diminished in the human uterus after the menopause Human cancers show increased beta glucuronidase activity compared with the tissue of origin

Amino sugars. Glucosamine and its N acetyl derivative are components of the group-specific agglutinogens of the red blood cells Acetylglucosamine also occurs in mucoitin sulfuric acid, hyaluronic acid, and heparin Galactosamine is characteristic of chondroitin sulfuric acid The L-enantiomorph of glucosamine is a structural component of the antibiotic, streptomycin D glucosamine is phosphorylated by ATP in beef brain extracts by the same enzymic mechanism which promotes the phosphorylation of glucose and fructose (27)

Little is known of the site or mechanism of the formation of amino sugars They and their condensation products are even more significant in forms lower than the mammals in the evolutionary series The chief supporting tissue of insects, crustaceans, and fungi is *chitin*, a polysaccharide composed of glucosamine The carbohydrate component of egg albumin has as its chief component *N* acetyl-3,4,6 trimethyl D glucosamine (56)

Formation of Fat from Sugars

The capacity of the body to store glycogen is sharply limited The liver storage is an amount equivalent to about 500 Kcal, and the maximal storage in the muscles about 2500 Kcal The total is scarcely adequate to meet the energic needs of one day of strenuous activity The only significant mechanism whereby carbohydrate can be stored indefinitely is by conversion to fat This transformation is demonstrated grossly by the fact that hogs can be fattened for market on a low fat high-carbohydrate diet It has been demonstrated more elegantly by measuring the rate of conversion of tagged glucose to fatty acids In order that fatty acids may be formed from sugars, the latter must be first degraded to two-carbon fragments, and some foodstuff must be simultaneously utilized to provide energy This latter requisite is implicit in the fact that fats yield approximately 9 Kcal per gram on complete combustion, whereas sugars yield only about 4 Kcal per gram The extra energy stored in fat must derive from the only possible source—the chemical energy of foodstuffs (see sec

tion on biosynthesis of lipids Chapter 13) The formation of two carbon fragments from sugars will be taken up in the next section which deals with catabolism of carbohydrates The conversion of two-carbon fragments to fatty acids is accelerated by insulin (7) It has long been recognized clinically that insulin in small doses was helpful in promoting the deposition of fat

THE CATABOLISM OF CARBOHYDRATE

Glucose which reaches muscular or other cells must travel by way of the extracellular fluid It is difficult and unrewarding to attempt the measurement in the human subject of the glucose content of tissue fluids Sufficient experimental evidence exists to enable us to make the inference that the glucose content of tissue fluids is very close to that of the blood, and that fluctuations in the glucose level of one will be closely followed by similar fluctuations in the other Glucose reaches the blood, and thence passes freely to the extracellular fluids from three sources (a) direct absorption of glucose from the alimentary tract, (b) glycogenolysis and (c) gluconeogenesis The quantity of glucose contributed to blood sugar by the breakdown of glycogen is normally the smallest, compared with the other two sources It becomes more significant in situations of acutely increased demand

Glycolysis

There is a series of reactions, involving phosphoric esters of glucose, fructose galactose and their split products which makes a limited amount of energy available from these sugars without utilization of molecular oxygen The intermediates end products and specific enzymes for this series of reactions have been identified in many different tissues in many different organisms The whole process has repeatedly been demonstrated in organisms ranging from bacteria and yeasts, through protozoa to all phyla of animals The series was first worked out in yeast and in frog and mammalian muscle This sequence of reactions is known as *glycolysis*, sometimes for emphasis as anaerobic glycolysis

The sequence of glycolytic reactions as far as the formation of fructose-6 phosphate has already been described under glycogenesis These steps have been summarized in table 29 The sequence can start in any tissue with glycogen glucose, or fructose Direct glycolysis of galactose is probably limited to liver, as is glycogenesis from galactose

The next step, which is the beginning of glycolysis proper, requires a second molecule of ATP, which gives up a terminal phosphate group becoming ADP The phosphate attaches irreversibly to fructose-6-phosphate, forming fructose 1,6-diphosphate (Harden Young ester (formula

An enzyme, *beta glucuronidase* catalyzes the synthesis and hydrolysis of numerous glucuronides including those of substances of extraneous origin such as menthol and borneol and those of physiological origin such as estriol and pregnanediol (19) High activity of this enzyme is observed in liver spleen lung endocrine tissues and leukocytes Some degree of activity is observed in all fresh tissues Uterine beta glucuronidase activity is sub normal in ovariectomized mice, but is increased by injections of natural or synthetic estrogens The activity is greatly diminished in the human uterus after the *menopause Human cancers show increased beta glucuronidase* activity compared with the tissue of origin

Amino sugars Glucosamine and its N acetyl derivative are components of the group-specific agglutinogens of the red blood cells Acetylglucosamine also occurs in mucoitin sulfuric acid, hyaluronic acid, and heparin Galactosamine is characteristic of chondroitin sulfuric acid The L-enantiomorph of glucosamine is a structural component of the antibiotic, streptomycin D glucosamine is phosphorylated by ATP in beef brain extracts by the same enzymic mechanism which promotes the phosphorylation of glucose and fructose (27)

Little is known of the site or mechanism of the formation of amino sugars They and their condensation products are even more significant in forms lower than the mammals in the evolutionary series The chief supporting tissue of insects, crustaceans, and fungi is *chitin*, a polysaccharide composed of glucosamine The carbohydrate component of egg albumin has as its chief component *N* acetyl-3,4,6 trimethyl D glucosamine (56)

Formation of Fat from Sugars

The capacity of the body to store glycogen is sharply limited The liver storage is an amount equivalent to about 500 Kcal, and the maximal storage in the muscles about 2500 Kcal The total is scarcely adequate to *meet the energic needs of one day of strenuous activity* The only significant mechanism whereby carbohydrate can be stored indefinitely is by conversion to fat This transformation is demonstrated grossly by the fact that hogs can be fattened for market on a low fat high carbohydrate diet *It has been demonstrated more elegantly by measuring* the rate of conversion of tagged glucose to fatty acids In order that fatty acids *may be* formed from sugars, the latter must be first degraded to two-carbon fragments, and some foodstuff must be simultaneously utilized to provide energy This latter requisite is *implicit in the fact* that fats yield approximately 9 Kcal per gram on complete combustion, whereas *sugars* yield only about 4 Kcal per gram The extra energy stored in fat must derive from the only possible source—the chemical energy of foodstuffs (see sec

tion on biosynthesis of lipids, Chapter 13) The formation of two carbon fragments from sugars will be taken up in the next section, which deals with catabolism of carbohydrates The conversion of two carbon fragments to fatty acids is accelerated by insulin (7) It has long been recognized clinically that insulin in small doses was helpful in promoting the deposition of fat

THE CATABOLISM OF CARBOHYDRATE

Glucose which reaches muscular or other cells must travel by way of the extracellular fluid It is difficult and unrewarding to attempt the measurement in the human subject of the glucose content of tissue fluids Sufficient experimental evidence exists to enable us to make the inference that the glucose content of tissue fluids is very close to that of the blood, and that fluctuations in the glucose level of one will be closely followed by similar fluctuations in the other Glucose reaches the blood, and thence passes freely to the extracellular fluids, from three sources (a) direct absorption of glucose from the alimentary tract, (b) glycogenolysis, and, (c) gluconeogenesis The quantity of glucose contributed to blood sugar by the breakdown of glycogen is normally the smallest, compared with the other two sources It becomes more significant in situations of acutely increased demand

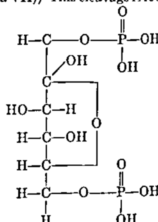
Glycolysis

There is a series of reactions, involving phosphoric esters of glucose, fructose, galactose, and their split products which makes a limited amount of energy available from these sugars without utilization of molecular oxygen The intermediates, end products and specific enzymes for this series of reactions have been identified in many different tissues in many different organisms The whole process has repeatedly been demonstrated in organisms ranging from bacteria and yeasts, through protozoa to all phyla of animals The series was first worked out in yeast and in frog and mammalian muscle This sequence of reactions is known as *glycolysis*, sometimes for emphasis as anaerobic glycolysis

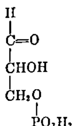
The sequence of glycolytic reactions as far as the formation of fructose 6 phosphate has already been described under glycogenesis These steps have been summarized in table 29 The sequence can start in any tissue with glycogen, glucose, or fructose Direct glycolysis of galactose is probably limited to liver, as is glycogenesis from galactose

The next step, which is the beginning of glycolysis proper, requires a second molecule of ATP, which gives up a terminal phosphate group becoming ADP The phosphate attaches irreversibly to fructose 6 phosphate, forming fructose 1,6-diphosphate (Harden-Young ester (formula

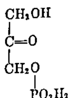
V)), commonly called hexose diphosphate or HDP The enzyme which catalyzes this phosphorylation is different from the hexokinases and is named *phosphohexokinase*, or better *phosphofructokinase* Fructose 1,6-diphosphate next cleaves into two triose phosphates glyceraldehyde-3 phosphate (Fischer Baer ester (formula VI)) and dihydroxyacetone phosphate (Kiessling ester (formula VII)) This cleavage is reversible and is specifically



V Fructofuranose 1 6 diphosphate
(Harden Young ester)



VI Glyceraldehyde 3 phosphate
(Fischer Baer ester)



VII Dihydroxyacetone phosphate
(Kiessling ester)

catalyzed by an enzyme known both as *aldolase* and as *zymohexase* The two isomeric triose phosphates are interconvertible and form an equilibrium mixture Their interconversion is catalyzed by *triose isomerase* Dihydroxyacetone phosphate has no further function in the glycolytic process It can, however, be enzymatically reduced to alpha glycerophosphate (Karrer ester), which is utilized in phospholipid synthesis and which can form glycerol upon hydrolysis catalyzed by a phosphatase

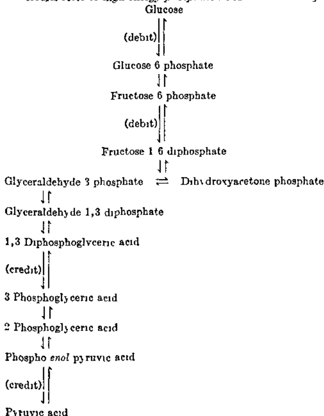
Glyceraldehyde-3 phosphate reacts spontaneously with inorganic phosphate to form glyceraldehyde 1 3 diphosphate (formula VIII) The metabolic cycle is now ready for the generation of a high-energy phosphate

bond Note that so far two such high energy bonds (table 30) have been used up by conversion of ATP to ADP, and that the bonds on the hexose and triose phosphates so far have all been low energy

TABLE 30

Summary of glycolysis

Debits and credits refer to high energy phosphate bonds utilized and produced

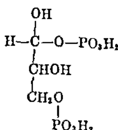


Two extra credits accrue per hexose unit (see text)

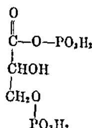
Glyceraldehyde 1 3 diphosphate is oxidized with the aid of *triosephosphate dehydrogenase*, by the transfer of hydrogen to the oxidized form of DPN (coenzyme I), which is thereby reduced The oxidation product of glyceraldehyde 1,3 diphosphate is 1,3 diphosphoglyceric acid (Negelein Bromel ester (formula IX)) The phosphate in the number 1 position is no longer a low-energy phosphoric acid acetal, but is now a high-energy

anhydride The high energy phosphate is next transferred to ADP, converting it to ATP The transfer is catalyzed by *phosphopherase* Since each hexose molecule has used two molecules of ATP to reach this stage and each triose molecule (two from each hexose) has generated one molecule of ATP from ADP, the books are now even in regard to high-energy phosphate bonds

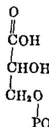
The 3 phosphoglyceric acid (Nilsson ester (formula X)), which remains after the delivery of the high-energy phosphate, rearranges catalyzed by *phosphoglyceromutase*, into 2 phosphoglyceric acid (Meyerhof Kiessling



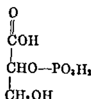
VIII Glyceraldehyde 1 3-diphosphate



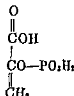
IX. 1 3 Diphosphoglyceric acid (Negelein Bromel ester)



X 3 Phosphoglyceric acid (Nilsson ester)



XI 2 Phosphoglyceric acid (Myerhof Kiessling ester)



XII Phospho-*enol* pyruvic acid (Lohmann Myerhof ester)

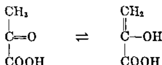
ester (formula XI)) This substance then loses water reversibly, in the presence of the enzyme *enolase*, to form phospho *enol* pyruvic acid (Lohmann Meyerhof ester (formula XII)) Here again a high energy phosphate bond is generated, the phosphate group starts as an ester of a secondary alcohol and finishes as a high-energy enol ester The phosphate can now be passed on by the aid of a specific transphosphorylase, to ADP converting it to ATP, the residue is pyruvic acid The ledger of high-energy phosphate bonds now shows a credit balance Each triose molecule has generated one extra high-energy bond, for each original hexose this makes two such bonds gained This is the end of the glycolytic process Hexose has been converted to pyruvic acid Each hexose molecule has used two high-energy phosphate bonds from ATP, and has returned four such bonds to ADP

Inhibitors of Glycolysis

The cytochrome oxidase system, which operates in aerobic oxidations, is inhibited by salts of hydrazoic acid, such as *sodium azide* (NaN_3) Azides also have the property of dissociating glycolysis from the transfer of phosphate bond energy Glycolysis continues but those cell activities which depend upon it for energy cease The uncoupling occurs while 1,3 diphosphoglyceric acid is still bound to phosphopherase (55) The chief inhibiting effect of *fluorides* upon glycolysis is exerted by action upon enolase This is indicated by the accumulation of 2 phosphoglyceric acid in fluorided preparations *Iodoacetic acid* inhibits triosephosphate dehydrogenase

Utilization of Pyruvic Acid

All animal cells and tissues as well as many plant cells and most of the familiar micro organisms use pyruvic acid both in synthetic processes and as a source of energy In alkaline solutions even of such low alkalinity



XIII Pyruvic acid

as prevails in animal tissues pyruvic acid exists as a keto-enol equilibrium mixture (formula XIII)) The existence of the two forms increases the reactivity of pyruvic acid It is able to take part in numerous condensations, dismutations, and oxido reductive reactions in the majority of which thiamine pyrophosphate or cocarboxylase is a necessary coenzyme

In the fermentation of hexoses by yeast, two additional steps occur in the glycolytic process Pyruvic acid loses CO_2 to form acetaldehyde, and the acetaldehyde is reduced to ethyl alcohol These steps are absent or insignificant in animal metabolism

In animal tissues reduction of pyruvic acid to *lactic acid* occurs under conditions of low oxygen concentration, since under such conditions DPN is available chiefly in the reduced form The reaction is catalyzed reversibly by *lactic acid dehydrogenase*, with oxidation of DPN Lactic acid is the end product of glycolysis, when the process is carried on under anaerobic conditions In this step, as in the preceding glycolytic steps, there is no utilization of molecular oxygen, nor formation of CO_2 Lactic acid is a metabolic blind alley in the animal organism It can only be reconverted to pyruvic acid or excreted In the brain, increased concentration of lactic acid and decreased concentration of high-energy phosphate has been observed in animals killed during hypoxic states Similar observations have

been made in animals in states of shock (see Chapter 15), in convulsions and following injury A purely emotional disturbance, such as fright (50), can bring about an increase in lactic acid even in rats treated with curare which prevents muscular production of excess lactic acid Brain lactic acid concentrations are decreased in rats during sleep

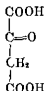
Lactic acid and pyruvic *acid coexist in the blood Since both are diffusible* substances, they presumably coexist in the cells in proportions similar to those in blood The average levels in the blood for a resting and fasting human subject are 8 2 mgm lactic acid and 0 78 mgm pyruvic acid per 100 ml of blood (29) Increases of both substances have been consistently demonstrated after mild exercise and after glucose ingestion

Transamination of pyruvic acid to alanine is accelerated by the enzyme transaminase in the presence of pyridoxal phosphate as coenzyme The amino group must come from an amino acid, which is converted by the transfer to a keto acid Isotopic studies indicate a small conversion of pyruvic acid to serine and glycine

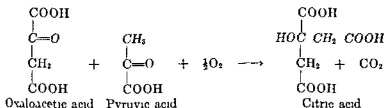
The tricarboxylic acid cycle (citric acid cycle or Krebs cycle) is a series of reactions in which the carbon atoms of pyruvic acid are oxidized one at a time to CO_2, with stepwise liberation of energy which is transferred to mechanical or chemical effectors by the generation of high-energy phosphate bonds Not only is the pyruvic acid derived by glycolysis of hexose sugars so oxidized with release of available energy, but so also are metabolic intermediates of lipids and proteins oxidized in the tricarboxylic acid cycle and energy similarly made available Such inclusiveness makes this oxidative mechanism singularly important, added to this we have the fact that the *cyclophorase* system of enzymes, which catalyzes the cycle, can be demonstrated in muscle, liver, brain, and kidney While complete *positive evidence is lacking for tissues other than those named,* there is quite general agreement that the tricarboxylic acid cycle is the chief mechanism for the oxidative utilization of foodstuffs as sources of energy This concept of the universality of the tricarboxylic acid cycle applies to all animals, including protozoa, and to yeast and *many molds and* aerobic bacteria The cycle will be presented here in summary form, a detailed discussion of the history of the concept, and the evidence upon which it has been established, has been given by Krebs in his Harvey Lecture (34)

The cycle begins and ends with oxaloacetic acid (formula XIV) This acid can be formed independently of the cycle by the addition of CO_2 to pyruvic acid (28), catalyzed by a soluble enzyme system present in liver and including biotin (see Chapter 19), and can also be formed by the oxidative deamination of aspartic acid (see Chapter 14) Once oxaloacetic acid is involved in the cycle, it is constantly regenerated and, in theory at least, calls for no independent source

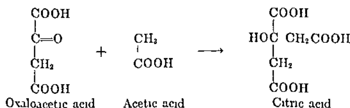
Oxaloacetic acid reacts with pyruvic acid to form citric acid (formula XV) (46) This reaction does not proceed in the reverse direction in animal tissues The equation represents the over all reaction, with no attempt to indicate intermediate stages The most probable intermediate is acetic acid (formula XVI), derived from pyruvic acid by oxidative decarboxylation Acetic acid adds to oxaloacetic acid as a carboxymethyl, $—CH_2COOH$, group (46), attached to phosphorylated coenzyme A (see Chapter 19) In



XIV Oxaloacetic acid



XV



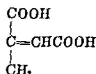
XVI

other reactions which will be considered in the next chapter, acetic acid may be introduced into biosynthetic reactions as the acetyl, $CH_3CO—$, group, also combined with coenzyme A These combinations of acetic acid with coenzyme A have been designated as "active acetate", carboxymethyl and acetyl are often spoken of collectively as "2 carbon fragments" Evidence gained from the use of isotopic carbon (24) indicates that only one of these 2 carbon fragments, namely carboxymethyl, can combine with oxaloacetic acid to form citric acid, thus initiating the tricarboxylic acid cycle

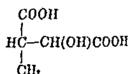
Note that in this reaction, unlike those of glycolysis, oxygen is required

and carbon dioxide is produced A necessary coenzyme is thiamine pyrophosphate In states of severe thiamine (vitamin B_1) deficiency, pyruvate concentrations in blood and urine increase as a result of inhibition of reactions involving the participation of pyruvic acid Pantothenic acid another B vitamin, *is also required*, in *the form of* coenzyme A These requirements are observed in isolated reactions The cyclophorase system contains all necessary enzymes and coenzymes High-energy phosphate bonds are necessary to initiate the cycle, and are regenerated in considerably greater numbers in the oxidative steps

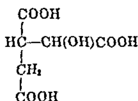
Wherever citric acid is found in biological systems, there also are two other tricarboxylic acids, *cis* aconitic acid (formula XVII) and isocitric



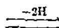
XVII Aconitic acid

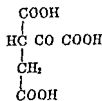


XVIII Isocitric acid



Isocitric acid





Oxalosuccinic acid

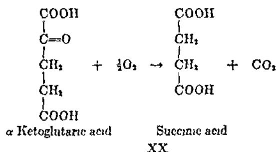
XIX

acid (formula XVIII) which form an equilibrium mixture There has been much controversy over which is formed first, and which is the one converted in the subsequent reaction Krebs originally proposed the order, citric → *cis* aconitic → isocitric, which was later modified on the basis of the interpretation of certain tracer experiments Reinterpretation of these experiments has permitted a return *to the original order*

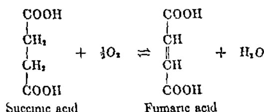
Isocitric acid is reversibly oxidized to oxalosuccinic acid (formula XIX) catalyzed by isocitric dehydrogenase, with reduction of the oxidized form of TPN

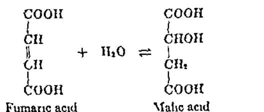
Oxalosuccinic acid is reversibly decarboxylated in the presence of Mn^{++} *and oxalosuccinic carboxylase*, to form CO_2 and alpha ketoglutaric acid which may leave the cycle by transamination to form glutamic acid, or may continue in the cycle by oxidative decarboxylation, apparently irreversible in animal tissues, to form succinic acid (formula XX) This makes

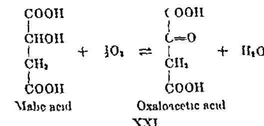
the third molecule of carbon dioxide, accounting for the three carbon atoms of pyruvic acid Succinic acid is now reconverted to oxaloacetic acid by the *dicarboxylic acid cycle* (formula XXI)



XX







XXI

The *cyclophorase system* is the group of enzymes and coenzymes necessary and sufficient to catalyze the reactions composing the tricarboxylic acid cycle The structural unit of the cyclophorase system is the mitochondrion Mitochondria contain protein, combined with phospholipid and with pen

tose nucleic acid When mitochondrial preparations are broken up mechanically numerous enzymes are liberated which catalyze individual reactions of the tricarboxylic acid cycle, *but the orderly sequence of the cycle as a whole* no longer is maintained In the normal operation of the cycle no accumulation of intermediates occurs, and each enzyme works effectively even at minute concentrations of substrate The entering materials appear to be taken up by the appropriate enzymes and then passed on in order to other enzymes which are probably located in definite positions on the surface of the mitochondrion Unlike the situation when enzymes act in solution, interaction between enzyme and substrate does not appear to depend upon random collision Most of the enzymes of the cyclophorase system appear to be pyridinoprotein enzymes Fresh mitochondrial preparations contain not only all the necessary enzymes but also all the necessary coenzymes for the catalysis of the Krebs cycle Older preparations require the pyridine nucleotides as well as adenosine 5-phosphate or its phosphorylated forms and Mg^{++} for maximal activity The function of thiamine and of pantothenic acid as part of the cyclophorase system has already been indicated

Oxidative phosphorylation in the course of the Krebs cycle requires the presence of intact mitochondria, and permits the storage in high-energy phosphate bonds of 60 per cent or more of the energy liberated by aerobic oxidations The mechanism of phosphate bond formation in the cyclophorase system has not been elucidated Certain landmarks have been charted by Green in (16)

Fixation of carbon dioxide When CO_2 or bicarbonate containing an isotopic carbon atom is administered to experimental animals, some of the carbon isotope finds its way into organic compounds of the animal's body Such incorporation of CO_2 takes place by three known reactions

1 CO_2 plus pyruvic acid yielding oxaloacetic acid,
2 CO_2 plus alpha ketoglutaric acid yielding oxalosuccinic acid
3 CO_2 plus ornithine, yielding citrulline (see page 536)

Undoubtedly other physiological reactions utilize CO_2 synthetically For example, tagged CO_2 appears in purines and pyrimidines in amounts not fully explicable by the reactions cited The appearance of isotopic carbon from CO in glycogen and glucose is explained as the result of gluconeogenesis from oxalosuccinic or oxaloacetic acids Isotopic carbon from these substances can appear in fats, since fats may be synthesized in the body from carbohydrate

DEFECTS OF CARBOHYDRATE METABOLISM IN DIABETES

Experimental diabetes in dogs was first produced by removal of the pancreas This was accomplished by *Mering and Minkowski* in 1889 The

organ fundamentally at fault in human diabetes mellitus is also the pancreas, specifically the beta cells of the islets of Langerhans (67) The sole known hormone produced by the beta cells is *insulin* Diabetes in the human can always be attributed either to an absolute deficiency of insulin or to a relative deficiency Relative deficiency may result from excessive activity of hormones antagonistic to insulin arising from the adrenal cortex anterior pituitary or thyroid, less commonly insulin may be inactivated by insulinase or by specific antibodies

The most striking manifestations of insulin deficiency are disturbances *of carbohydrate utilization glycosuria and hyperglycemia The metabolism* of fat is also highly abnormal with failure to convert sugar to fat, and failure to oxidize fatty acids completely Diabetic acidosis or *ketosis*, results from the accumulation of acetoacetic acid and its derivatives beta hydroxybutyric acid and acetone, acetoacetic acid is a normal intermediate in the oxidative utilization of fatty acids The lipids of the blood, including fats, phospholipids, and cholesterol, are *increased Protein metabolism* particularly protein synthesis is disturbed with excessive gluconeogenesis from protein the chief abnormality Although we formally classify diabetes as a disease of carbohydrate metabolism this distinction is historical and somewhat artificial It is best considered as a disease of metabolism in general

Insulin is a protein hormone, with a molecular weight of about 48,000 at pH 7, in acid solutions it dissociates into fragments of molecular weight 12 000 and below pH 3 of molecular weight 6 000 (23) which are still physiologically active In alkaline solutions insulin quickly loses activity Proteolysis destroys activity, chymotrypsin for example catalyzes the cleavage of insulin into peptides of molecular weight about 800 each plus an inactive protein residue with 80 per cent of the original cystine On account of the destructive action of some of the proteolytic digestive enzymes, insulin effects are minimal and unreliable when the drug is taken by mouth The subcutaneous route is ordinarily used, the intravenous occasionally

Banting and Best produced in 1921 the first successful insulin by ligation of the pancreatic ducts of dogs, causing atrophy of the externally secreting cells which produce proteolytic and other digestive enzymes and leaving only the islands of Langerhans This precluded the destructive action of proteolytic enzymes which had previously prevented the extraction of insulin in water solution Large scale production avoids this maneuver, whole pancreases from animals slaughtered for meat are extracted with strongly acidified alcohol The extract is salted out with ammonium sulfate and the insulin precipitated at its isoelectric point (about pH 5)

Insulin is assayed in units, three units will depress the blood sugar of a

fasting 2 kgm rabbit to 0 045 per cent Pure crystalline zinc insulinate contains 22 units per mgm The dosage of insulin for each patient is a matter of *individual study for his physician* The dosage is established, with the patient on a constant diet, by blood sugar measurements before and after insulin administration Since there is no direct chemical or mathematical relationship between units of insulin administered and grams of carbohydrate metabolized, attempts at determining and utilizing such a relationship are fallacious and may be dangerously misleading Overdosage of insulin produces hypoglycemia, with accompanying weakness, tremor, sweating, and often *unconsciousness Relief of hypoglycemia is by admin*istration of sugars, preferably glucose, by mouth if the patient is conscious, intravenously if the patient is unconscious A 20-gram dose of glucose by either route is usually adequate

Insulin in human blood has been measured by bioassay on alloxan diabetic (see page 510) hypophysectomized adrenalectomized rats (9) Fasting human blood contains about 0 1 unit of insulin per liter, increasing to as much as 0 34 unit per liter after glucose ingestion The normal human pancreas contains about 2,000 units per kilogram, the average diabetic pancreas about 20 per cent of this figure Application of the same bioassay technique to the blood plasma of human diabetics divides such patients *into two distinct groups One group has no demonstrable free plasma* insulin, the other has a normal amount

The Functions of Insulin

According to the concept of insulin function proposed by the Cori group one important function of insulin is to abolish the effect of pituitary, adrenocortical, and possibly other inhibitors of glucokinase Insulin acts to promote the formation of glucose 6 phosphate, to lower the level of blood glucose, to increase glycogen formation both in liver and in muscle, and to accelerate the glycolysis of glucose These effects occur after the administration of insulin—they are facts which are explained by the Cori concept but which are independent of the future complete confirmation of the Cori concept, or its possible future modification or abandonment

Certain other actions of insulin seem less closely related to the release

the citric acid cycle when these functions are depressed, as they a experimental animals made diabetic by the injection of alloxan (25) In normal cats, insulin increases the incorporation of radioactive P^{32} into ATP and into phosphocreatine (51) Insulin promotes the formation of fatty acids from two carbon fragments, and favors the building of proteins from amino acids (37)

Insulin forms a chemical combination with muscle (rat diaphragm) and simultaneously increases the rate of glucose uptake and glycogen synthesis of the muscle If the rat is previously rendered diabetic by alloxan (see page 510) or if the rat is previously injected with either crude anterior pituitary extract or purified pituitary growth hormone, the ability of the muscle to combine with insulin is diminished (58) Even *in vitro* crude pituitary extracts diminish the insulin-combining power of muscle specimens, but purified growth hormone does not

Considerable experiment and discussion has been lavished upon the question whether diabetes is the result of *underutilization* of glucose in the tissues or of *overproduction* of glucose by excessive gluconeogenesis and hepatic glycogenolysis It is now apparent that both mechanisms are involved Insulin administered to the diabetic both increases tissue utilization and checks hepatic output of glucose The more fundamental effect appears to be that upon tissue utilization Whether by the release of glucokinase from inhibition or by some other mechanism insulin promotes the uptake of glucose by tissue cells The uptake of fructose does not appear to require insulin The measurement of cellular uptake of sugars has been accomplished by the measurement of the simultaneous decrease in plasma inorganic phosphate, which is required in the phosphorylation processes accompanying cellular uptake A dog rendered diabetic by total pancreatectomy shows no change in tolerance for fructose as compared with a normal dog The expected decrease in plasma inorganic phosphate occurs following fructose injection Following glucose injection into such a pancreatectomized dog, no fall in plasma inorganic phosphate is observed unless insulin is supplied (36), or unless glucose is administered in greatly increased concentration and over longer periods of time, thus forcing its entry into tissue cells These experiments neither confirm nor exclude the Cori concept, but clearly demonstrate that insulin is necessary for the uptake of glucose by tissue cells at physiological levels of blood glucose

Insulin and the Anterior Pituitary

A hypophysectomized dog can be killed promptly by the same dose of insulin which would be well tolerated by a normal dog Diabetes produced in dogs by removal of the pancreas is mitigated following hypophysectomy Such pancreatectomized and hypophysectomized dogs (called 'Houssay dogs' after the pioneer experimenter in this field) develop severe diabetes after injection of anterior pituitary extracts In rats removal of the pituitary produces a 50 per cent increase in muscle hexokinase activity (3), with a further increase of similar magnitude upon administration of insulin

Hyperglycemia and glycosuria can be induced in adult intact cats and dogs by the daily administration for 3 to 8 days of Young's (70) anterior

pituitary diabetogenic factor Preparations of the diabetogenic factor have not been obtained free from growth hormone Glycosuria has been produced in cats by purified preparations of pituitary growth hormone The initial response to daily injections of the diabetogenic pituitary extract is an increase in body weight with retention of nitrogen Glycosuria appears after several days *The increase in weight is not the result of water retention* but is associated with synthesis of muscle protein This is in contrast to the failure of protein synthesis usually observed in human diabetes Young has not been able consistently to evoke diabetic symptoms in puppies and kittens by the use of his extracts which are effective in adult dogs and cats Since 1938 there has been discussion and study of the question of the participation of insulin in the normal growth response to pituitary somatotrophic hormone The status of this still unresolved problem has been summarized by Evans (18)

Young differentiates *idiohypophyseal* diabetes which is the immediate result of injection of pituitary diabetogenic substance from *metahypophyseal diabetes which persists after a period of such injections and is* characterized by degranulation hydropic degeneration and hyalinization of the beta cells of the islets Animals with metahypophyseal diabetes can survive for a long time without extraneous insulin but their actual insulin requirement is greater than that of comparable totally depancreatectomized animals This is probably the result of the continued presence of the alpha cells of the pancreatic islets which secrete a substance antagonistic to insulin (see page 505) It still remains to be demonstrated that Young's diabetogenic factor has any effect directly upon hexokinase

The anterior pituitary is concerned with adaptation to starvation It retards sugar utilization and increases gluconeogenesis Fasting intact rats *economize their carbohydrate reserves showing scarcely any* change in the total glycogen of the body between 24 and 48 hours of fasting Hypophysectomized fasting rats ... bohydrate at a rat ... times more rapid than that of the n... ...g rat (11) Insul... ...rned with adaptation to food int... ...s storage of f... glycogen as fat a... prot... ...tion with in... sugar

The g... ...ent the ... effects of the ante... ...ge d... ...roduces tempora... t... terized ficiency *other cort* genesis

Patients curve whic

from the intestine There is however, an actual decrease in the rate of glycogen formation from intravenously injected glucose (67) This effect of thyroid overactivity has not been adequately explained in terms of known mechanisms of glucose utilization

Insulinase is an enzyme system identified in liver kidney, and muscle Both endogenous and injected insulin are inactivated by insulinase (3) Insulin injected into the portal vein of dogs is less effective than insulin injected into the femoral vein Liver insulinase is decreased during fasting and restored by feeding

Antibodies which neutralize the hormonal action of insulin have been demonstrated in a small proportion of rabbits after repeated injection, and arise spontaneously in a small proportion of insulin resistant human diabetics following treatment (38) Allergy to injected insulin as manifested by itching at the site of injection or by urticaria is more frequently related to species specific animal protein than to organ-specific insulin Allergic manifestations can sometimes be obviated by the use of more highly purified insulin or insulin prepared from a different species The antibody involved in allergic responses is not identical with the antibody which neutralizes hormone action Patients with insulin allergy typically show the normal metabolic effects of insulin

A *pancreatic hyperglycemic hormone* (glukagon) is secreted by the alpha cells of the islets of Langerhans It is a protein, and functions by promoting hepatic glycogenolysis It may be that glycogen storage disease (see page 487) results from a lack of this hormone This contrary acting substance is present in small amount in most available preparations of insulin

THE DIABETIC PATIENT

The diabetic is likely to have ancestors or blood relatives who are diabetics The evidence bearing upon the inheritance of diabetes has been summarized by Joslin and his associates (32) At the time when the disease first becomes manifest the patient is likely to be overweight With progress of the disease weight is usually lost as a result of the loss of potential calories by glycosuria Weakness occurs proportional to the weight loss

Diabetics particularly if inadequately treated, are susceptible to infections Staphylococcal infections of the skin are far more threatening to the diabetic than to the normal person Tuberculous infection is likely to become active in the diabetic

Carotenemia Diabetics often show a pigmentation of the skin, often a yellowish color of serum much greater than normal This has been shown to result from the accumulation of several lipochrome pigments, particularly carotene Oral administration of carotene to diabetics results in a higher blood level of carotene, maintained for a greater length of time than

in non-diabetics The assumption has been made that the liver in diabetes is unable to convert carotene into vitamin A as rapidly as normal hence the accumulation of carotene in blood and tissues

Arteriosclerosis, which involves lipid infiltration followed by calcification of the arterial walls, is perhaps a disease or perhaps a part of the normal process of ageing In diabetics it appears earlier and progresses faster than in the general population. In many diabetics it is a major complication and may lead to death from vascular occlusion Arteriosclerosis begins as an abnormality of lipid metabolism and as such will be considered in the next chapter There is usually a high blood lipid content in severe diabetes Cholesterol is elevated in the blood of untreated diabetics, the elevation often persists after treatment Since high blood cholesterol levels are statistically correlated with the development of arteriosclerosis, the use of low fat diets is being more and more recommended

Ketosis is the most urgent complication of diabetes and may lead quickly to coma and death It is a serious failure of fat oxidation, and causes a severe acidosis It will be discussed *in these two aspects* in the appropriate chapters In brief ketosis is the failure to oxidize fats completely An intermediate metabolic product, acetoacetic acid together with its reduction product, beta hydroxybutyric acid and its decarboxylation product acetone accumulate in the body and appear in the urine *The resulting coma is in part* the result of increased blood and tissue acidity and in part a direct result of the action of high concentrations of these ketone bodies ' on the nervous system Dehydration and loss of ions from body fluids, both the result of vomiting and polyuria, add to the dangers of diabetic ketosis The most effective prevention and treatment of diabetic ketosis is by the use of insulin Ketosis also occurs as a result of starvation in which case food is required and insulin is *contraindicated* Note for the present that both situations in which ketosis may develop involve a subnormal rate of glucose utilization

Fertility of untreated diabetic women is low The chief *contributing* factor is fetal or neonatal death Diabetic mothers show low levels of estrogens and of pregnanediol excretion and high levels of *chorionic gonado*trophin The fetus is frequently oversized and edematous

Principles of diabetic treatment Our present state of knowledge offers no means for the permanent cure of diabetes The physician treating a diabetic patient attempts to maintain a reasonably *normal blood sugar* level avoiding hypoglycemia and excessive hyperglycemia (over 0 15 per cent) He also tries to keep the patient's body weight normal *He plans to* protect the patient from ketosis which is the cause of diabetic coma, and if possible from arteriosclerosis which is the direct or indirect cause, since the discovery of insulin of most diabetic deaths

Education of the patient concerning the nature of the disease the means available for its control and particularly the hopeful outlook for the properly controlled patient is perhaps the most important feature of successful treatment The disease demands full time attention, and few patients can afford the full time services of a medical attendant Fortunately, the procedures which are the daily necessities of the diabetic are not too complicated to be learned by the child who can comprehend his school work or the adult who is capable of earning a living In medical centers, diabetic education is carried on in organized classes Under other circumstances, the instruction is individualized and given by the physician or a competent assistant

Diet is the most important daily variable to be brought under control Restriction of food intake has been an important part of the treatment of diabetes for as long as the metabolic nature of the disease has been recognized Such dietary restriction is usually voluntary on the part of the patient encouraged and advised by his physician At times it has been enforced by circumstances entirely out of the control of either During the periods of food rationing necessitated in Britain and in Germany by World Wars I and II, striking decreases in death rates from diabetes occurred The British figures also show smaller decreases in diabetic mortality corresponding to the two periods of economic depression between these wars (41)

The cardinal principle of the voluntary dietary treatment of diabetes is to avoid overfeeding but the diabetic diet must be adequate in all the dietary essentials It is impossible to write down an arbitrary diet suitable for all diabetics It is important, particularly if insulin is being used, that the diet be constant from day to day as far as total calories from carbohydrate and carbohydrate forming foods are concerned It is customary to have food portions weighed at least until the patient is accustomed to the diet and can estimate weights with the help of common household measures The scrupulous weighing of the diet to the nearest gram may become exasperating during the protracted lifetime of the properly treated diabetic The degree of latitude which can or should be allowed to an individual patient in this respect depends so much upon the personalities of the patient his family and his physician, that no definite rules can be laid down

The complete elimination of *carbohydrates* from the diabetic diet is unwise Reduction of carbohydrate alone favors ketosis—a serious and too often fatal overloading of the mechanisms of fat oxidation The diabetic diets of a decade ago allowed 50 to 150 grams of carbohydrate per day, while present-day diets usually contain 150 to 300 grams In the low carbohydrate diets, the total calories must be made up by protein and fat

In practice, they were usually made up by fat, with increased danger of ketosis and arteriosclerosis *Protein* should not be restricted below the normal requirement of a healthy person, approximately 1 gram per kilogram of body weight per day Increase to 1 25 grams per kilogram is probably advantageous but adds to the expense of the diet Since protein is a little over half convertible to carbohydrate the extra protein calories may be deducted half from fat and half from carbohydrate The greater protein needs of growing children should be met as in health *Fat allowance bears some relation to the patient's nutritional state*, 50 grams per day is an almost standard figure It can be decreased for the obese diabetic or increased if it is otherwise difficult to maintain normal weight The vitamin and mineral requirements, particularly in children, may demand the use of vitamin concentrates and of milk

Exercise is a means of lowering blood sugar by increasing its utilization The effect is considerable in active children, so that unusual or protracted exercise may cause a child who is taking insulin to develop hypoglycemia Just as a diabetic's diet and insulin should be measured daily, so should his exercise be as constant as possible from day to day Additional exercise should be compensated by extra carbohydrate or possibly less insulin Patients require less insulin upon discharge from the hospital and resumption of activity Study of blood levels of lactic and pyruvic acids in diabetic patients indicates that these substances are formed more slowly after glucose ingestion than in the normal subject The response of the diabetic patient to exercise is however, normal in regard to the production of lactic and pyruvic acids Insulin appears to be necessary for glycolysis in the resting subject, but not for glycolysis during exercise (29) Physiological experimentation thus confirms clinical experience that exercise can to a degree at least substitute for insulin

Insulin may not be required by mild diabetics, and in fact is usually not prescribed if the patient can tolerate a diet adequate in calories and containing *150 grams of carbohydrate without consistent glycosuria* There is considerable variance among specialists in diabetes in their manner of use of insulin, and in their attitude towards strict control of diet and exercise A procedure acceptable to many authorities, and widely used, is to start an adult patient on an adequate measured diet, as described with a small dose of protamine zinc insulin (about 12 units) injected before breakfast *Protamine zinc insulin* is insulin modified with 1 25 mgm of protamine derived from fish sperm and 0 2 mgm of zinc for each 100 units of insulin Protamine zinc insulin differs from regular insulin, which has an action lasting twelve hours, with peak activity at between three and four hours, by having its action spread out in time by the slow rate of dissociation of the compound of insulin with protamine Protamine zinc insulin has a low peak of

activity at twenty four hours or more, so that the minimum blood sugar level will be just before breakfast, following the longest fasting period of the twenty four hours Since glycosuria is dependent upon blood sugar level the urine at that time should contain the least sugar The dose of protamine zinc insulin is increased, usually 4 units a day until the early morning urine is free from sugar In most cases the blood sugar, taken before breakfast, will then be normal or not greatly elevated The urine and blood sugars are

[illegible]

increase of the single dose of protamine zinc insulin would lead to hypo glycemia in the early hours of the morning Instead, regular insulin is given ahead of the protamine insulin and thirty minutes before breakfast Again, the dose is small at the start usually 8 units and is increased daily by 4 units until the blood sugar is normal four hours after the injection If urine is now sugar free and blood sugar is not over 0 150 per cent one hour after the noon meal the dosage is adequate If not a second dose of regular insulin is demanded thirty minutes before the noon meal, which is increased as before checking blood and urine sugar one hour after the evening meal In some cases, three injections of regular insulin may be needed *Globin insulin* and *NPH insulin* are intermediate in duration of activity between regular and protamine zinc insulin Patients who require two or more doses of regular insulin in addition to the single dose of protamine zinc insulin can often be carried on a single dose of globin or NPH insulin Regular insulin can be added to NPH insulin without serious loss of the promptness of action of the regular insulin, permitting a single injection daily even for those patients who require additional regular insulin (15)

NON-DIABETIC MELITURIAS

The demonstration of reducing sugars in the urine by Benedict's or a similar test does not of itself indicate diabetes *Glucose* may occur in the urine of a non-diabetic as a result of (a) heavy ingestion of sugars, (b) emotional stress (c) hyperthyroidism (d) hyperactivity of anterior pituitary or adrenal cortex (but this type of glycosuria may have many points in common with diabetes) (e) intracranial damage, particularly of the hypothalamic region, from trauma vascular accident, infection or tumor and (f) as a concomitant of severe infections, intoxications, and chronic diseases see Joslin (32) for elaboration of this last rather indefinite class *Lactose* occurs in the urine post partum and during lactation *Pentose* (L-ketoxylose or L-xylulose) or *fructose* may be found in the urine in rare cases of metabolic anomaly

Renal glycosuria is distinct from these categories and from diabetes Glu

In practice, they were usually made up by fat with increased dangers of ketosis and arteriosclerosis. *Protein* should not be restricted below the normal requirement of a healthy person, approximately 1 gram per kilogram of body weight per day. Increase to 1.25 grams per kilogram is probably advantageous, but adds to the expense of the diet. Since protein is a little over half convertible to carbohydrate, the extra protein calories may be deducted, half from fat and half from carbohydrate. The greater protein needs of growing children should be met as in health. *Fat* allowance bears some relation to the patient's nutritional state; 50 grams per day is an almost standard figure. It can be decreased for the obese diabetic or increased if it is otherwise difficult to maintain normal weight. The vitamin and mineral requirements, particularly in children, may demand the use of vitamin concentrates and of milk.

Exercise is a means of lowering blood sugar by increasing its utilization. The effect is considerable in active children, so that unusual or protracted exercise may cause a child who is taking insulin to develop hypoglycemia. Just as a diabetic's diet and insulin should be measured daily, so should his exercise be as constant as possible from day to day. Additional exercise should be compensated by extra carbohydrate or possibly less insulin. Patients require less insulin upon discharge from the hospital and resumption of activity. Study of blood levels of lactic and pyruvic acids in diabetic patients indicates that these substances are formed more slowly after glucose ingestion than in the normal subject. The response of the diabetic patient to exercise is, however, normal in regard to the production of lactic and pyruvic acids. Insulin appears to be necessary for glycolysis in the resting subject but not for glycolysis during exercise (29). Physiological experimentation thus confirms clinical experience that exercise can to a degree at least substitute for insulin.

Insulin may not be required by mild diabetics, and in fact is usually not prescribed if the patient can tolerate a diet adequate in calories and containing 150 grams of carbohydrate without consistent glycosuria. *There is* considerable variance among specialists in diabetes in their manner of use of insulin and in their attitude towards strict control of diet and exercise. A procedure acceptable to many authorities, and widely used, is to start an adult patient on an adequate measured diet, as described, with a small dose of protamine zinc insulin (about 12 units) injected before breakfast. *Protamine zinc insulin* is insulin modified with 1.25 mgm. of protamine derived from fish sperm and 0.2 mgm. of zinc for each 100 units of insulin. Protamine zinc insulin differs from regular insulin, which has an action lasting twelve hours with peak activity at between three and four hours, by having its action spread out in time by the slow rate of dissociation of the compound of insulin with protamine. Protamine zinc insulin has a low peak of

tivity at twenty four hours or more, so that the minimum blood sugar level will be just before breakfast, following the longest fasting period of the twenty four hours Since glycosuria is dependent upon blood sugar level, the urine at that time should contain the least sugar The dose of protamine zinc insulin is increased usually 4 units a day until the early morning urine is free from sugar In most cases the blood sugar, taken before breakfast, will then be normal or not greatly elevated The urine and blood sugars are then measured one hour after the noon meal If the urine is sugar free and

its until the blood sugar is normal four hours after the injection If urine is now sugar free and blood sugar is not over 0 150 per cent one hour after the noon meal the dosage is adequate If not a second dose of regular insulin is demanded thirty minutes before the noon meal, which is increased as before, checking blood and urine sugar one hour after the evening meal In some cases three injections of regular insulin may be needed *Globin insulin* and *NPH insulin* are intermediate in duration of activity between regular and protamine zinc insulin Patients who require two or more doses of regular insulin in addition to the single dose of protamine zinc insulin can often be carried on a single dose of globin or NPH insulin Regular insulin can be added to NPH insulin without serious loss of the promptness of action of the regular insulin, permitting a single injection daily even for those patients who require additional regular insulin (15)

NON-DIABETIC MELITURIAS

The demonstration of reducing sugars in the urine by Benedict's or a similar test does not of itself indicate diabetes *Glucose* may occur in the urine of a non-diabetic as a result of (a) heavy ingestion of sugars (b) emotional stress (c) hyperthyroidism (d) hyperactivity of anterior pituitary or adrenal cortex (but this type of glycosuria may have many points in common with diabetes) (e) intracranial damage, particularly of the hypothalamic region, from trauma, vascular accident, infection, or tumor and (f) as a concomitant of severe infections, intoxications and chronic diseases (see Joslin (32) for elaboration of this last rather indefinite class *Lactose* occurs in the urine post partum and during lactation *Pentose* (l-ketoxylose or l-xylulose) or fructose may be found in the urine in rare cases of metabolic anomaly

Renal glycosuria is distinct from these categories and from diabetes Glu

In practice, they were usually made up by fat, with increased danger of ketosis and arteriosclerosis *Protein* should not be restricted below the normal requirement of a healthy person, approximately 1 gram per kilogram of body weight per day Increase to 1 25 grams per kilogram is probably advantageous but adds to the expense of the diet Since protein is a little over half convertible to carbohydrate, the extra protein calories may be deducted, half from fat and half from carbohydrate The greater protein needs of growing children should be met as in health *Fat* allowance bears some relation to the patient's nutritional state, 50 grams per day is an almost standard figure It can be decreased for the obese diabetic or increased if it is otherwise difficult to maintain normal weight The vitamin and mineral requirements, particularly in children, may demand the use of vitamin concentrates and of milk

Exercise is a means of lowering blood sugar by increasing its utilization The effect is considerable in active children, so that unusual or protracted exercise may cause a child who is taking insulin to develop hypoglycemia Just as a diabetic's diet and insulin should be measured daily, so should his exercise be as constant as possible from day to day Additional exercise should be compensated by extra carbohydrate or possibly less insulin Patients require less insulin upon discharge from the hospital and resumption of activity Study of blood levels of lactic and pyruvic acids in diabetic patients indicates that these substances are formed more slowly after glucose ingestion than in the normal subject The response of the diabetic patient to exercise is however, normal in regard to the production of lactic and pyruvic acids Insulin appears to be necessary for glycolysis in the resting subject, but not for glycolysis during exercise (29) Physiological experimentation thus confirms clinical experience that exercise can to a degree at least substitute for insulin

Insulin may not be required by mild diabetics, and in fact is usually not prescribed if the patient can tolerate a diet adequate in calories and containing 150 grams of carbohydrate without consistent glycosuria There is considerable variance among specialists in diabetes in their manner of use of insulin and in their attitude towards strict control of diet and exercise A procedure acceptable to many authorities, and widely used, is to start an adult patient on an adequate measured diet, as described, with a small dose of protamine zinc insulin (about 12 units) injected before breakfast *Protamine zinc insulin* is insulin modified with 1 25 mgm of protamine derived from fish sperm and 0 2 mgm of zinc for each 100 units of insulin Protamine zinc insulin differs from regular insulin, which has an action lasting twelve hours, with peak activity at between three and four hours, by having its action spread out in time by the slow rate of dissociation of the compound of insulin with protamine Protamine zinc insulin has a low peak of

activity at twenty four hours or more, so that the minimum blood sugar level will be just before breakfast, following the longest fasting period of the twenty four hours Since glycosuria is dependent upon blood sugar level, the urine at that time should contain the least sugar The dose of protamine zinc insulin is increased usually 4 units a day until the early morning urine is free from sugar In most cases the blood sugar, taken before breakfast,

increase of the single dose of protamine zinc insulin would lead to hypoglycemia in the early hours of the morning Instead, regular insulin is given ahead of the protamine insulin, and thirty minutes before breakfast Again the dose is small at the start usually 8 units and is increased daily by 4 units until the blood sugar is normal four hours after the injection If urine is now sugar free and blood sugar is not over 0 150 per cent one hour after the noon meal, the dosage is adequate If not a second dose of regular insulin is demanded thirty minutes before the noon meal, which is increased

can often be carried on a single dose of globin or NPH insulin Regular insulin can be added to NPH insulin without serious loss of the promptness of action of the regular insulin, permitting a single injection daily even for those patients who require additional regular insulin (15)

NON-DIABETIC MELITURIAS

The demonstration of reducing sugars in the urine by Benedict s or a similar test does not of itself indicate diabetes *Glucose* may occur in the urine of a non-diabetic as a result of (a) heavy ingestion of sugars (b) emotional stress (c) hyperthyroidism (d) hyperactivity of anterior pituitary or adrenal cortex (but this type of glycosuria may have many points in common with diabetes) (e) intracranial damage particularly of the hy

occurs in the urine post partum and during lactation *Pentose* (L-ketoxylose or L-xylulose) or fructose may be found in the urine in rare cases of metabolic anomaly

Renal glycosuria is distinct from these categories and from diabetes Glu

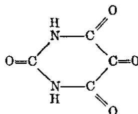
In practice they were usually made up by fat, with increased danger of ketosis and arteriosclerosis *Protein* should not be restricted below the normal requirement of a healthy person, approximately 1 gram per kilogram of body weight per day Increase to 1 25 grams per kilogram is probably advantageous but adds to the expense of the diet Since protein is a little over half convertible to carbohydrate, the extra protein calories may be deducted half from fat and half from carbohydrate The greater protein needs of growing children should be met as in health *Fat* allowance bears some relation to the patient's nutritional state, 50 grams per day is an almost standard figure It can be decreased for the obese diabetic or increased if it is otherwise difficult to maintain normal weight The vitamin and mineral requirements, particularly in children, may demand the use of vitamin concentrates and of milk

Exercise is a means of lowering blood sugar by increasing its utilization The effect is considerable in active children, so that unusual or protracted exercise may cause a child who is taking insulin to develop hypoglycemia Just as a diabetic s diet and insulin should be measured daily, so should his exercise be as constant as possible from day to day Additional exercise should be compensated by extra carbohydrate or possibly less insulin Patients require less insulin upon discharge from the hospital and resumption of activity Study of blood levels of lactic and pyruvic acids in diabetic patients indicates that these substances are formed more slowly after glucose ingestion than in the normal subject The response of the diabetic patient to exercise is however, normal in regard to the production of lactic and pyruvic acids Insulin appears to be necessary for glycolysis in the resting subject, but not for glycolysis during exercise (29) Physiological experimentation thus confirms clinical experience that exercise can to a degree at least, substitute for insulin

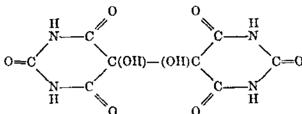
Insulin may not be required by mild diabetics, and in fact is usually not prescribed if the patient can tolerate a diet adequate in calories and containing 150 grams of carbohydrate without consistent glycosuria There is considerable variance among specialists in diabetes in their manner of use of insulin and in their attitude towards strict control of diet and exercise A procedure acceptable to many authorities and widely used is to start an adult patient on an adequate measured diet, as described with a small dose of protamine zinc insulin (about 12 units) injected before breakfast *Protamine* [illegible] derived from f [illegible] mine [illegible] twelve hours with peak activity at between three and four hours by having its action spread out in time by the slow rate of dissociation of the compound of insulin with protamine Protamine zinc insulin has a low peak of

hours, and finally, (c) permanent hyperglycemia The period of hypoglycemia is pancreatic in origin, probably the result of liberation of preformed insulin by the degenerating beta cells

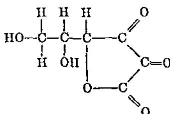
So far alloxan has been useful as an additional means of producing experimental diabetes Some of the facts learned by its use may in time help



XXII Alloxan



XXIII Alloxantin



XXIV Dehydroascorbic acid

elucidate the cause of ordinary human diabetes For example, glutathione is protective against destruction by alloxan of the beta cells, as well as against the production of diabetes by ACTH Blood glutathione decreases along with successful induction of alloxan diabetes Animals with depleted blood glutathione are more susceptible to alloxan Dehydroascorbic acid (formula XXIV) may be considered as having a structure not unlike alloxan One injection of 1 1 grams per kgm body weight will produce hyperglycemia in rats Three daily injections will produce permanent diabetes Sulfhydryl compounds such as cysteine and glutathione, if given intravenously two

cose is excreted *in the urine at normal levels of blood sugar* as a result of failure of the renal tubule cells to reabsorb glucose from the glomerular filtrate. Such a situation is often designated as a "lowered glucose threshold" in the kidney. For the time, we can state that the normal renal threshold for glucose is about 0.16 per cent. Above this blood sugar level glycosuria occurs, below it there is no glycosuria. This is a crude and oversimplified statement, but is usually true.

Renal glycosuria is of fairly frequent occurrence accompanying diseases such as nephrosis where there is degeneration of renal tubule cells. In many instances, however, it occurs as an isolated phenomenon, with no symptoms and no impairment of nutrition or general health. The glycosuria may be constant or periodic. A moderate degree of renal glycosuria is frequently observed during pregnancy, disappearing after delivery, and returning with further pregnancies. Renal glycosurics may develop diabetes, but the existence of renal glycosuria does not increase the probability of developing diabetes. The differentiating observation between diabetes and renal glycosuria is the blood sugar—high in diabetes, normal in renal glycosuria. In borderline cases, the glucose tolerance test is usually decisive. Patients with uncomplicated renal glycosuria require no active treatment. Restriction of diet is not helpful, and may result in undesirable undernutrition. It is standard practice to check blood sugars at regular intervals throughout such a patient's lifetime.

The *Fanconi syndrome* is a more serious failure of renal tubular absorption in which there is not only failure to reabsorb sugar, resulting in glycosuria, but also failure to reabsorb amino acids, Ca^{++}, HPO_4^{--}, and water, resulting in growth failure, rickets, and polyuria. It is characteristically a disease of children, rickets and failure of growth usually appearing at about the second year of life.

Galactose diabetes (4) is a rare but serious familial inability to metabolize galactose. It causes severe malnutrition, which can be avoided by substituting other foods for milk. The disease is quite comparable to diabetes in that there is hypergalactemia combined with galactosuria, and fatty infiltration of the liver.

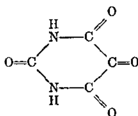
ALLOXAN DIABETES

Certain substances injected intravenously will cause destruction of the beta cells of the islets of Langerhans. These substances include alloxan (formula XXII) and alloxantin (formula XXIII).

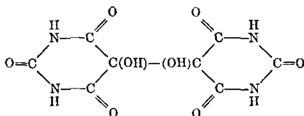
Methyl alloxan and dimethyl alloxantin are similarly effective. Alloxan has a similar effect when administered by *other routes*. The characteristic response of the experimental animal to injection of alloxan is (a) hyperglycemia lasting one hour or less, then (b) hypoglycemia lasting several

hours and finally, (c) permanent hyperglycemia The period of hypoglycemia is pancreatic in origin, probably the result of liberation of preformed insulin by the degenerating beta cells

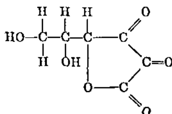
So far alloxan has been useful as an additional means of producing experimental diabetes Some of the facts learned by its use may in time help



XXII Alloxan



XXIII Alloxantin



XXIV Dehydroascorbic acid

elucidate the cause of ordinary human diabetes For example, glutathione is protective against destruction by alloxan of the beta cells as well as against the production of diabetes by ACTH Blood glutathione decreases along with successful induction of alloxan diabetes Animals with depleted blood glutathione are more susceptible to alloxan Dehydroascorbic acid (formula XXIV) may be considered as having a structure not unlike alloxan One injection of 1 1 grams per kgm body weight will produce hyperglycemia in rats Three daily injections will produce permanent diabetes Sulfhydryl compounds such as cysteine and glutathione, if given intravenously two

minutes before the injection of dehydroascorbic acid will prevent its di[illegible] betogenic action If given ten minutes afterwards the sulfhydryl com[illegible] pounds have no protective effect For this reason Patterson and Lazaro (44) conclude that dehydroascorbic acid irreversibly blocks the sulfhydr[illegible] groups of some important enzyme

SPONTANEOUS HYPOGLYCEMIA

Low levels of blood sugar comparable to those observed after insul[illegible] overdosage sometimes occur when insulin is not given The symptoms ar[illegible] similar to those of insulin overdosage In mild cases the patient is weak hungry and uneasy often showing blurring of vision perspiration tremor [illegible] drunken! or collapse S c[illegible]

Langerhans, and (c) diseases of the liver

Reactive hyperinsulinism is an exaggerated physiological response to in creased blood sugar levels It is likely to occur in conscientious persons wh[illegible] are hyperreactive to everyday frustrations It has also been observed i[illegible] patients who have been submitted to gastroenterostomy or total or subtota[illegible] gastric resection The latter group represents the result of too sudden a[illegible] entrance of carbohydrate into the absorptive portion of the small intestine Both groups tend to have hypoglycemic attacks two to four hours afte[illegible] meals, and do well on a high protein low carbohydrate diet

Islet cell tumors or hyperplasia lead to relatively constant insulin output most notably indicated by low fasting blood sugar levels in contrast to th[illegible] [illegible] ve type Patients with tumor o[illegible]

[illegible] a hypoglycemic state comparabl[illegible] to that observed in experimental animals after hepatectomy The obviou[illegible] cause is inability of the severely damaged liver to carry on the functions o[illegible] glycogenesis glycogenolysis and particularly gluconeogenesis In this situa tion treatment is exactly opposite to that of reactive hypoglycemia Sinc[illegible] hypoglycemia occurs after relatively long periods of fasting meals should be frequent and should be high in carbohydrate The protein content of the diet should be governed by the nature and stage of the hepatic disease In the majority of diseases involving destruction of liver cells a high pro tein allowance is advantageous This will be discussed further in Chapter 14

METABOLISM OF ALCOHOL

[illegible]

venous administration of alcohol to surgical patients [illegible]

operative day (49), or longer if indicated Alcohol is given usually in 5 per cent solution in water or isotonic salt solution containing 5 per cent glucose At this concentration, and at a rate of 15 ml alcohol (300 ml of 5 per cent solution) per hour, a moderate analgesic and sedative drug effect of alcohol is achieved together with the supply of caloric needs both by alcohol and by glucose

When alcohol tagged with C^{14} was administered to rats in dosages of 1 gram per kgm of body weight (2), 75 per cent of the C^{14} was recovered as CO_2 within five hours and 90 per cent in ten hours Rats habituated to drinking 10 per cent alcohol instead of water for five months showed no increase in the rate of alcohol oxidation Tissue slices of rat liver and kidney converted alcohol to CO_2 rapidly, heart and diaphragm oxidized alcohol slightly, and brain not at all

Liver contains an alcohol dehydrogenase which catalyzes the conversion of ethyl alcohol to acetaldehyde, with DPN as the hydrogen acceptor Acetaldehyde is converted to acetic acid catalyzed by an aldehyde dehydrogenase Acetic acid is oxidized by way of the citric acid cycle Alcohol, upon complete oxidation yields 7 Kcal per gram There is no evidence that the energy liberated in the conversion of alcohol to acetic acid is available for performance of work via phosphate bond formation The oxidation of acetic acid from alcohol in the citric acid cycle is strictly comparable to the combustion of acetic acid from other sources, is phosphate linked, and is a source of energy

The early stages of oxidation of alcohol in the human body proceed at a limited rate which is reasonably constant in each individual In different individuals the rate varies so that the blood alcohol concentration falls by 0 01 to 0 02 per cent per hour Two ounces of 100 proof whiskey taken by a person of average size will build up a maximal concentration of alcohol in the blood of about 0 05 per cent Concentrations below this level produce no evidences of intoxication except in a few unusually susceptible subjects Six ounces or more of 100 proof whiskey can raise the blood alcohol concentration above 0 15 per cent, at which level all subjects show clear evidence of impairment of neuromuscular function Blood levels of 0 15 per cent or above have been accepted in courts of law as *prima facie* evidence of alcoholic intoxication in cases involving violation of motor vehicle laws At blood alcohol levels between 0 05 per cent and 0 15 per cent there can usually be demonstrated by laboratory methods some degree of delay in reactions involving judgment or discrimination, and some subjects are definitely and obviously intoxicated Alcohol concentrations can be measured directly on blood samples by a distillation method or more conveniently by estimation from the alcohol content of expired air (31) Blood alcohol concentrations of the order of 0 5 per cent are lethal

REFERENCES

1 Artz, N E, and Osman, E M *Biochemistry of Glucuronic Acid* New York, Academic Press, 1950.

2 Bartlett, G R, and Barnet, H N Some observations on alcohol metabolism with radioactive ethyl alcohol Quart J Studies on Alcohol, 10: 381-397, 1949

3 Beaser, S B Diabetes Mellitus New England J Med, 243. 81-85, 1950

4 Bell, L S, *et al* Lesions of galactose diabetes Arch Path, 49. 393-403, 1950

5 Benedict, S R, and Osterberg, E A method for the determination of sugar in normal urine J Biol Chem, 48: 51-57, 1921

6 Bhishagratna, K L *An English Translation of the Sushruta Samhita*, I. p 75 Calcutta, India, Wilkins Press, 1907

7. Bloch, K, and Kramer, W Effect of pyruvate and insulin on fatty acid synthesis *in vitro* J Biol Chem, 173: 811-812, 1948

8 Bonnichsen, R K, and Wassen, A M Crystalline alcohol dehydrogenase from horse liver. Arch Biochem, 18: 361 362, 1948

9 Bornstein, J. Normal insulin concentration in man Australian J Exper Biol & Med Sc, 28: 93-97, 1950

10 Conn, J W. The diagnosis and management of spontaneous hypoglycemia J A M A, 134: 130-138, 1947

11 Cori, C F Enzymatic reactions in carbohydrate metabolism Harvey Lect, Series XLI, 253-272 1946

12 deBodo, R C, *et al* Loss of insulin hypersensitivity and development of diabetes *in hypophysectomized dogs produced by purified growth hormone* Proc Soc Exper Biol & Med, 74 524-526 1950

13 Dickens, E, and Glock, G E Direct oxidation of glucose-6 phosphate by animal tissues Nature, 166: 33, 1950

14 Drury, H F Identification and estimation of pentoses in the presence of glucose Arch Biochem, 19: 455-466, 1948

15 Duncan, G G *Diabetes Mellitus* Philadelphia, Penna, W B Saunders Co, 1951

16 Edsall, J T *Enzymes and Enzyme Systems* Cambridge, Mass, Harvard University Press, 1951

17 Enklewitz, M, and Lasker, M The origin of L xyloketose (urine pentose) J Biol Chem, 110. 443-456, 1935

18 Evans, H M The search for the diabetogenic principle of the anterior hypophysis Proc Am Diabetes Assoc, 9: 17-28, 1949.

1 [illegible] *The Enzymes*, Vol I, Part [illegible]

[illegible]

21 Fc [illegible]

1920.

22 Fosdick, L S, and Rapp, G W The degradation of glucose by *Staphylococcus albus* Arch Biochem, 1: 379-389, 1942

23 Fredericq, E, and Neurath, H The interaction of insulin with thiocyanate and other anions, The minimum molecular weight of insulin J Am Chem Soc, 72: 2684-2691, 1950

24 Geyer, R P, *et al* Inhibition studies on fatty acid metabolism J Biol Chem, 188. 185-191, 1951

25 GORANSSON E S AND TULLKAR S D The effect of insulin on the aerobic phos phorylation of creatine in tissues from alloxan diabetic rats Arch Biochem 24 40-48 1949

26 GRIFFITHS M The mechanism of the diabetogenic action of uric acid J Biol Chem 184 289-298 1950

27 HARPUR R P AND QUASTEL J H Relations between acetylcholine synthesis and metabolism of carbohydrates and D glucosamine in the central nervous system Nature 164 779-782 1949

28 HASTINGS A B et al Incorporation of isotopic carbon dioxide in rabbit liver glycogen in vitro J Biol Chem 177 71 26 1949

29 HORWITT M K et al Lactic and pyruvic acids in the blood after glucose and exercise in diabetes mellitus Am J Physiol 156 92-99 1949

30 JACOBSEN E Is acetaldehyde an intermediary product in normal metabolism? Biochem et Biophys Acta 4 330-334 1950

31 JETTER W W A critical survey of various chemical methods for determining the alcohol content of body fluids and tissues with their physiological and medico legal significance Quart J Studies on Alc hol 2 512 543 1941

32 JOSLIN E P et al The Treatment of Diabetes Mellitus 8th edit Philadelphia Lenna Lea and Febiger 1946

33 KEENE D L Effect of alloxan pancreatectomy and adrenalectomy on plasma amino nitrogen as studied by means of hemorrhage Am J Physiol 154 87 93 1948

34 KREBS H A The tricarboxylic acid cycle Harvey Lect 1948-49

35 LARDY H A, ed Respiratory Enzymes Minneapolis Minn Burgess Publishing Co 1949

36 LEVINE R et al Nature of the action of insulin on the level of serum inorganic phosphate Am J Physiol 159 107 110 1949

37 LOTSPEICH W D The role of insulin in the metabolism of amino acids J Biol Chem 179 175-180 1949

38 LOWELL F C AND FRANKLIN W Induced insulin resistance in the rabbit J Clin Investigation 28 199-206 1949

39 MALPRESS F H AND MORRISON A B Studies on the synthesis of lactose by the mammary gland I Precursors Biochem J 46 307 312 1950

40 MANN T AND LARDON L Studies on the metabolism of semen Biochem J 46 440-450 1950

41 MEAKINS J C The Practice of Medicine 5th edit St Louis Mss Mosby 1950

42 MOSBACH E H AND KING C G Tracer studies of glucuronic acid biosynthesis J Biol Chem 185 491-498 1950

43 NAJJAR V A The isolation and properties of phosphoglucomutase J Biol Chem 175 281 290 1948

44 PATTERSON J W AND LAZAROW A Sulfhydryl protection against dehydroascorbic acid diabetes J Biol Chem 186 141 144 1950

45 PIGMAN W W AND GOEPP R M JR Chemistry of the Carbohydrates New York Academic Press 1948

46 POTTER V R AND HEIDELBERGER C Alternative metabolic pathways Physiol Rev 30 487-512 1950

47 RAPPORT D et al Conversion of hexose to pentose by mammalian tissue Am J Physiol 162 421-427 1950

48 RENVALL S Oxidation pathway of the D glucose dehydrogenase Acta chem scandinav 4 738-743 1950

49 Rice C O, *et al* Parenteral nutrition in the surgical patient as provided from glucose, amino acids, and alcohol The role played by alcohol Ann Surg, **131**: 289-306, 1950
50 Richter, D, and Dawson, R M C Brain metabolism in emotional excitement and in sleep Am J Physiol, **154**. 73-79, 1948
51 Sacks, J The effect of insulin on phosphorus turnover in muscle Am J Physiol, **143**: 157-162, 1945
52 Sarton, G *Introduction to the History of Science* Vol I, p 76 Baltimore, Md, The Williams & Wilkins Co, 1927
53 Shaffer, P A, and Somogyi, M Copper iodometric reagents for sugar determination J Biol Chem, **100**. 695-713, 1933
54 Smith, R H, and Williams Ashman, H G Influence of thyroxine on hexokinase, succinoxidase, and choline oxidase Nature, **164** 457-458, 1949
55 Spiegelman, S, *et al* *Phosphate metabolism and the dissociation of anaerobic glycolysis from synthesis in the presence of sodium azide* Arch Biochem **18**. 409-436, 1948
56 Stacey, M The chemistry of mucopolysaccharides and mucoproteins Advances in Carbohydrate Chem, **2**. 161-201, 1946
57 Stadie, W C, *et al* The effect of insulin and adrenal cortical extract on the *hexokinase reaction in extracts of muscle from depancreatized cats* J Biol Chem, **184** 617-626, 1950
58 Stadie, W C, *et al* Hormonal influences on the chemical combination of insulin with rat muscle (diaphragm) Am J Med Sc, **218** 275-280, 1949
59 Stetten, D, Jr The study of certain pathologic processes with the aid of isotopic hydrogen New York State J Med, **47**: 1991-1996, 1947
60 Stetten, D, Jr Carbohydrate metabolism Am J Med 7. 571-584 1949
... acid in
J Biol
Chem, **65** 393-395, 1925
63 Teply, L J Studies on the cyclophorase system XIV Mechanism of action of
64 ... 7-146 1948
65
66 Werner, I On the regeneration of serum polysaccharide and serum proteins in normal and intoxicated rabbits Acta physiol scandinav, **19** 27-42 1949
67 Wilder, R M Reflections on the causation of diabetes mellitus J A M A, **144**. 1234-1239, 1950
68 Wood, H G The fixation of carbon dioxide and the interrelationships of the tricarboxylic acid cycle Physiol Rev, **26** 198-246, 1946
69 Work, T S, and Work, E *The Basis of Chemotherapy* New York, Interscience Publishers, 1948
70 Young, F G The relationship of the anterior pituitary gland to diabetes mellitus Acta med scandinav, **135**: 275-288, 1949

CHAPTER 13

Lipid Metabolism and Ketosis

The fats and related lipids distinguish themselves among the foodstuffs by their high caloric value Each gram of fat yields 9 3 Kcal, which is more than twice the energy yield (4 1 Kcal) of a gram of carbohydrate or protein The amount of fat included in human dietaries is highly variable, but usually remains within the limits of 50 and 150 grams per day if circumstances permit free choice Small amounts of certain unsaturated fatty acids appear to be necessary in human nutrition (see Chapter 10)

BLOOD LIPIDS

According to Thannhauser (25), normal human blood plasma may contain from zero to 200 mgm of neutral fat per 100 ml from 150 to 250 mgm of phospholipids, chiefly phosphoglycerides, and from 150 to 200 mgm of cholesterol 70 to 75 per cent of which is in the form of fatty acid esters and the remaining 40 to 70 mgm is free or unesterified The plasma lipids are almost entirely combined in the form of lipoprotein The figures cited refer to plasma collected before breakfast in the normal subject Values in excess of these may be observed following the ingestion of lipid rich meals, or as a result of fasting prolonged beyond the usual overnight period, or after protracted exercise The lipomicrons and chylomicrons which can be seen in the blood after lipid rich meals are plasma beta lipoprotein combined with extra triglyceride (18) Hyperlipemia or abnormal increase of neutral fat in blood plasma is a characteristic pathological change in severe diabetes in nephrosis, in certain types of anemia, and following the administration of alcohol and other anesthetic drugs There is also a familial derangement of fat metabolism designated as essential hyperlipemia Milkiness of the plasma is noted when hyperlipemia is excessive, but Thannhauser (25) has observed increases in neutral fat up to 150 per cent of normal which were detectable only by chemical analysis

Blood cholesterol increases with hyperlipemia and with hypoproteinemia

There is a very high blood cholesterol in untreated diabetes decreasing with insulin therapy, but not often receding to the normal level The blood cholesterol is typically elevated in hypothyroidism

BODY LIPIDS

Lipids in the body originate from ingested lipids, and by the transformation of carbohydrate and protein into lipid material Every cell of the body contains lipids which are a part of the cellular structure (see Chapter 3) Such *tissue lipid* remains intact regardless of diminished caloric intake Phospholipids are important components of the cellular lipid In addition there is *storage lipid*, composed chiefly of neutral fats, and making *up the adipose tissue* Certain locations in the body function as *depots for* fat about half of the stored fat is found in the subcutaneous layer the remainder is divided into perirenal mesenteric omental, and subperitoneal deposits with a minimal amount in the fascial planes between the muscles

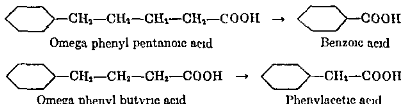
The fatty acids of human subcutaneous fat are as follows according to Calandra and Cattaneo (14) with the percentage of the total fatty acids given for each oleic 38 7 linoleic 24 8 palmitic, 20 8, eicosadienoic 3 3 palmitoleic, 3 2 gadoleic 2 8 stearic, 2 2 erucic 1 8 myristic 1 5 arachidonic and myristoleic 0 4 each arachidic 0 1 In addition to triglycerides of the above fatty acids there is about one per cent of cholesterol Each animal species has a characteristic composition of storage fat which is constant if the animal is fed chiefly upon carbohydrate and protein with a minimum of dietary fat If any animal is fed upon a high fat diet his depot fat will show a variation in composition in the direction of the composition of the fat which has been fed Fatty acids which are not normally found in the storage fat of the animal will occur there under these circumstances Stored fat is not metabolically inert, but is in constant exchange with blood lipids Hence abnormalities of the composition of storage fat will gradually disappear upon cessation of excessive fat feeding Similar alterations in fatty acid composition of milk fats can be observed in lactating animals fed upon high fat diets All cells including those of adipose tissue (21) contain lipases and esterases which facilitate the hydrolysis and resynthesis of lipids

Atherosclerosis is an accumulation of lipids in the walls of arteries and arterioles It is common after mid life is probably encouraged by a diet high in lipids and is certainly promoted by chronic hyperlipemia *as in* diabetes (18) Cholesterol bearing lipid and lipoprotein macromolecules have been demonstrated (13) by ultracentrifugal studies of the serum of men and of cholesterol fed rabbits associated with *atherosclerosis* in both species The concentration of such macromolecules could be diminished in human subjects by restriction of dietary fat and cholesterol No cor

relation exists, however, between the cholesterol content of the diet and the level of cholesterol in the blood plasma A diet low in cholesterol will bring about a temporary decrease in plasma cholesterol, which returns to its previous level within less than a year (10) The gradual return of the plasma cholesterol to its usual value must be attributed to an increased rate of cholesterol synthesis within the body Lipidoses are disturbances of lipid metabolism within tissue cells Several such metabolic diseases exist, and are discussed in detail by Thannhauser (25)

OXIDATION OF FATS

Glycerol can be phosphorylated and enter the glycolytic series of reactions, and therefore can form glucose and glycogen Fatty acids, in order to be oxidized in the Krebs cycle (see page 496), must first be converted to water soluble, two carbon fragments which can react with oxaloacetic acid to form citric acid The oxidation of the two carbon fragments then

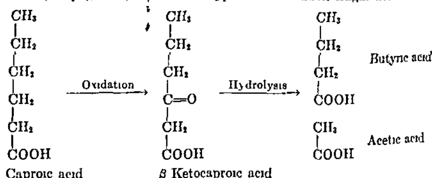


I

proceeds by the same tricarboxylic acid cycle described for carbohydrate Isotopically tagged carbon atoms of administered fatty acids appear in the acids which compose the tricarboxylic acid cycle The differences in oxidative mechanisms of fats as compared with carbohydrates occur, therefore, in the earlier steps It is important to recall, in considering these early processes, that the nutritionally significant fatty acids all have an even number of carbon atoms

It has been known for nearly half a century that the carbon atoms are removed from fatty acids in multiples of two Knoop reported in 1904 that animals would remove carbon atoms in pairs from the straight chains of omega phenyl substituted fatty acids Regardless of the length of the chain, the compound would be broken down in the animal body and the terminal phenyl group would be excreted either as benzoic acid or as phenylacetic acid (formula I), in either case conjugated with glycine (see page 541) If the chain had an odd number of carbon atoms, benzoic acid would be the end product, if there was an even number of carbon atoms, phenylacetic acid would be formed On the basis of this evidence, Knoop postulated the theory of beta-oxidation of fatty acids, which stated that an oxidative

attack is made on the beta carbon atom resulting in the splitting off of acetic acid and the formation of a new fatty acid shorter by two carbon atoms than the original Thus the beta oxidation of caproic acid would split off acetic acid and leave butyric acid (formula II) Longer fatty acids would be degraded *two carbon atoms at a time* in a similar manner Current biochemical opinion supported by the results of experiments with tagged fatty acids, still accepts the concept of beta oxidation but with certain modifications *It is no longer possible to consider that the two carbon fragment split off is simple acetic acid* The terminal two-carbon fragments of fatty acids are converted to acetyl CH_3CO—, groups which attach to coenzyme A The *non terminal two-carbon fragments form carboxy methyl groups* —CH_2COOH identical with those from pyruvic acid (see page 497) These two types of two carbon fragments can con



Caproic acid β Ketocaproic acid

II

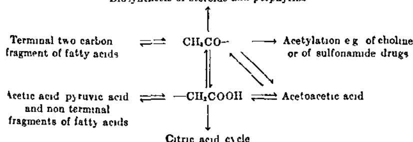
dense one of each to form acetoacetic acid (19) They are also to a degree interconvertible The possibilities of metabolic utilization of two-carbon fragments is shown in table 31

Oxidative degradation of fatty acids occurs in the liver down to the stage of a four carbon acid (butyric acid) from which acetoacetic acid can be formed by direct beta oxidation Acetoacetic acid appears not to be rapidly or effectively metabolized by liver cells but being water-soluble can enter the circulation and be utilized by extra hepatic tissues particularly muscle and kidney Octanoic acid is oxidized by rat liver preparations yielding two molecules of acetoacetic acid But if oxaloacetate is present less than two molecules of acetoacetic acid appear for each molecule of octanoic acid which disappears Instead the carbon which would have been expected to appear as acetoacetic acid is diverted to such compounds as citric acid alpha ketoglutaric acid *succinic acid and* CO_2 Added acetoacetic acid does not form such compounds hence it is concluded that the two carbon fragments which are detached from the fatty acid during beta

oxidation may either condense together to form acetoacetic acid or with oxaloacetic acid to enter the tricarboxylic acid cycle. In the body as a whole, however, tagged acetoacetic acid yields tagged citric acid, alpha ketoglutaric acid and fumaric acid. By varying the experimental conditions it is possible to set up systems which will oxidize acetic acid and not acetoacetic acid, or vice versa. It is improbable that either one is a necessary step in the oxidation of the other. The most probable conclusion is that acetic acid, acetoacetic acid, and fatty acids in general can all be degraded to the two carbon fragments which are the actual reactants with oxaloacetic acid. Within the liver, long chain fatty acids (e.g., palmitic) are more completely oxidized, forming more CO_2 and less acetoacetic acid, than are shorter chain acids (e.g., octanoic) (28).

TABLE 31

Metabolic utilization of the two-carbon fragments, acetyl (CH_3CO—), *and carboxymethyl* (—CH_2COOH), *(modified from Potter and Heidelberger (19))*



Preparations of mitochondria, obtained from rat liver by methods described by Lehninger (7), are highly effective catalysts for the aerobic oxidation of fatty acids as well as for the reactions of the Krebs tricarboxylic acid cycle. Coupled with both of these oxidative processes are oxidative phosphorylations, also catalyzed by the mitochondria. The individual enzymes catalyzing individual steps in these oxidative sequences have resisted isolation. The enzymes which compose the fatty acid oxidase system are particularly firmly bound to the mitochondrion, and continue to function with no apparent loss of activity after repeated resuspensions and washings. The activity of the mitochondrial fatty acid oxidase system *in vitro* requires the presence of ATP and of Mg^{++}, and furthermore requires "sparking" or "priming" by the presence of some intermediate in the Krebs cycle, such as fumaric acid or alpha ketoglutaric acid. Such an intermediate need be present only in catalytic amount to start fatty acid oxidation, which then maintains itself and regenerates the necessary intermediates.

A dehydrogenase system which acts upon higher fatty acids has been identified in the livers of several mammalian species (3) This system requires DPN and is inhibited *in vitro* by low concentrations of oleic acid Functions of a fatty acid dehydrogenase might well include (1) the preliminary stage of beta oxidation, and (2) the introduction or removal of a double bond The latter process is actually known to be carried out in the liver with certain limitations Both saturation and desaturation of fatty acids can be demonstrated in the liver, desaturation being usually predominant One double bond can be introduced into a saturated fatty acid in the liver with no apparent difficulty Introduction of further double bonds seems to be impossible The introduction of a double bond into saturated fatty acids is not a function of liver only, but has also been demonstrated in adipose tissue (29) So far, emphasis has been placed upon the liver as the chief organ involved in the early stages of fatty acid oxidation Experiments with eviscerated rats (12) indicate that free fatty acids are effectively and completely oxidized by tissues other than liver

Another type of fatty acid oxidation, in which the terminal methyl group is oxidized to carboxyl, has been demonstrated in intact men and dogs (26) Such *omega-oxidation* takes place most notably with saturated fatty acids not more than 12 nor less than 8 carbon atoms in length. The feeding of such a fatty acid leads to the appearance in the urine of dicarboxylic acids with either the same number of carbon atoms as the original fatty acid, or less than that number by multiples of two It therefore is evident that dicarboxylic acids within the 8 to 12 carbon atom range can undergo beta oxidation with splitting off of two carbon fragments This concept has been confirmed by the direct feeding of dicarboxylic acids to dogs and recovering them in the urine along with other dicarboxylic acids shorter by 2 or 4 carbon atoms The output of dicarboxylic acids in the urine, on a constant fatty acid intake, is increased by increasing the carbohydrate of the diet This is in contrast with the urinary output of ketone acids, which will be shown later to be diminished or abolished by adequate carbohydrate intake

Conversion of fatty acids to carbohydrate does not occur in animals as a significant physiological process One clear but little used pathway exists for this conversion—the formation of succinic acid by omega-oxidation and subsequent beta-oxidation Succinic acid, like the other four carbon dicarboxylic acids, is definitely convertible to glucose The evidence in regard to gluconeogenesis from fatty acids can be summarized in two main points (1) tagged carbon atoms administered in fatty acids do appear in glucose and glycogen, but (2) no net gain in glucose or glycogen follows the feeding of fatty acids to starving or diabetic animals To explain these apparently contradictory facts in terms of known oxidative mechanisms,

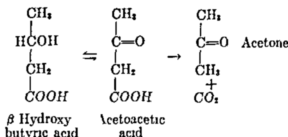
we must presume that there is no significant direct conversion of two-carbon fragments to pyruvic acid although this reaction is demonstrably possible and is so indicated by a double arrow in Table 31. Numerous other anabolic pathways are open to the two carbon fragment, but none of these leads to carbohydrate formation. When a two carbon fragment enters its only known catabolic reaction initiating the Krebs cycle by condensation with oxaloacetic acid carbohydrate forming intermediates are produced but two carbons are oxidized. The two carbons which are oxidized are not those of the two carbon fragment therefore tagged carbons of two carbon fragments derived from fatty acids may appear later in carbohydrates derived from Krebs cycle intermediates but since two other carbons have been oxidized there can result no net gain of carbohydrate.

KETOGENESIS AND KETOSIS

Acetoacetic acid can be reduced in liver to beta hydroxybutyric acid. These two are known clinically as the *ketone acids*. Acetoacetic acid will slowly decompose spontaneously to acetone and CO_2 (formula III). This reaction is not *reversible* but acetone can be metabolized to a limited extent (22 26). The ketone acids plus acetone make up the group of substances known clinically as the *ketone bodies* which are normal intermediates in fatty acid metabolism. Accumulation of the ketone bodies in tissues and body fluids in abnormal amounts is known as *ketosis* and most commonly results from starvation or from diabetes mellitus. In either of these situations oxidation of carbohydrate is deficient in the liver and fat becomes the only substance or the major portion of the material being oxidized in the liver. Ketosis is diminished or abolished by supplying carbohydrate in cases of starvation or by facilitating the utilization of carbohydrate in diabetes by the use of insulin. Clinical experience has taught us that fatty acids, plus certain amino acids which are metabolized in a manner comparable to fatty acids (see Chapter 14) are ketogenic, which means that they increase the production of ketone bodies. Carbohydrate glycerol and the majority of amino acids are *antiketogenic* which means that the metabolism of these substances diminishes the tendency to excessive ketone body production. Even in normal subjects it is possible to induce ketosis by feeding a diet in which the ketogenic substances are present in great excess over the antiketogenic substances. Such a *ketogenic diet* is of value in the treatment of epilepsy particularly in children for reasons which are not fully understood. Other therapeutic applications of the ketogenic diet such as in urinary tract infections have been superseded by more effective measures. If it should be desired to prescribe a ketogenic diet or conversely to prescribe a diet which would minimize the formation of ketone bodies the Schaffer K A ratio still remains a valuable check on

the ketogenic potentialities of a diet even though the theory upon which it was based is now known to be oversimplified In this formula K refers to ketogenic substances and A to antiketogenic substances (formula IV) If the value of this ratio for any diet exceeds 2 that diet is ketogenic and a person subsisting on that diet will probably show *ketonemia* (more than one mgm of ketone bodies, expressed as acetone, per 100 ml of blood) and *ketonuria* (the elimination of more than 20 mgm of ketone bodies expressed as acetone in the 24 hour urine)

Why are fatty acids ketogenic and carbohydrates antiketogenic? Krebs (16) points out that each two carbon fragment from carbohydrate requires one molecule of oxygen in its formation The same is true in the oxidation



III

$$\frac{K}{A} = \frac{(2\,4 \times \text{grams protein})\ (3\,43 \times \text{grams fat})}{(3\,2 \times \text{grams protein})\ (0\,57 \times \text{grams fat})\ (5\,56 \times \text{grams carbohydrate})}$$

IV

of a long chain fatty acid until a four carbon fragment is reached From the four carbon fragment two two carbon fragments are formed with the utilization of only one molecule of oxygen Metabolities are ketogenic if one molecule of oxygen can produce two two carbon fragments they are antiketogenic if they produce less than two two carbon fragments per molecule of oxygen In brief, the theory expounded by Krebs is that carbohydrate successfully competes with fatty acid for the available oxygen

The theory just summarized implies that carbohydrate is oxidized in preference to fatty acids and that the oxidation of carbohydrate or of carbohydrate intermediates would inhibit the oxidation of fatty acid In experiments designed to test this implication (27) no inhibition of the oxidation in rat liver slices of octanoic acid or of shorter fatty acids was induced by carbohydrate and in some instances the rate of fatty acid oxidation was increased The investigators concluded that the antiketogenic action of carbohydrate was exerted on the two carbon fragments derived

by beta-oxidation Carbohydrate can supply the oxaloacetic acid with which the two-carbon fragments must presumably condense to enter the tricarboxylic acid cycle The oxaloacetic acid may arise by the union of CO_2 with *pyruvic acid* (*see page 496*)

If, on the other hand fatty acid oxidation is studied in the intact rat (11), and palmitic acid, which is more than twice the length of octanoic acid, is used carbohydrate feeding does decrease the rate of oxidation of the fatty acid This finding does not contradict the work described in the preceding paragraph, since even in the intact rat the rate of oxidation of octanoic acid was little affected by carbohydrate intake Adequate evidence is still lacking to explain fully the ketogenic action of fatty acids and the antiketogenic action of glycerol and carbohydrates

Ketonuria A state of clinical ketosis is most commonly confirmed by the finding of one or more of the ketone bodies in the urine The common nitroprusside tests for acetone in the urine are really tests for acetoacetic acid To be sure, they will indicate the presence of acetone but they are at *least five times more sensitive to acetoacetic acid* When *urine specimens* are allowed to stand acetoacetic acid continues to break down into acetone and CO_2 which renders the nitroprusside tests less responsive to the same original amount of ketone bodies The most satisfactory of the numerous nitroprusside procedures is that of Rothera (9) 5 ml of fresh urine is saturated with solid ammonium sulfate and mixed with 10 drops of freshly prepared 2 per cent sodium nitroprusside solution, this is then mixed with 10 drops of concentrated ammonia water and allowed to stand 15 minutes The presence of acetoacetic acid or of larger concentrations of acetone, is indicated by the characteristic blue purple color

Diabetic ketosis is of common occurrence in diabetics who are untreated or inadequately treated The Rothera test is sensitive to low concentrations of acetoacetic acid in the urine, and may be positive in cases where the *patient shows no symptoms referable to ketosis A positive test for aceto* acetic acid in the urine should not, however be ignored Symptoms often develop rapidly and proceed to coma Pre coma symptoms and signs include anorexia nausea vomiting abdominal pain, increased pulmonary ventilation and drowsiness These symptoms arise in part from acidosis (see Chapter 16) and in part from toxic effects of the ketone bodies (8) There is often an acetone odor on the breath Treatment in this stage consists of increased insulin dosage or a re-evaluation of the patient s diet, usually in the direction of increased carbohydrate—or both In cases of actual coma an emergency exists and treatment is best carried on in a hospital For details of a rational and successful program for the treatment of diabetic coma see Duncan (6)

BIOSYNTHESIS OF LIPIDS

We can safely assume that the knowledge of the fattening of animals on diets relatively low in fat was put to use by our early pastoral ancestors In 1860 Lawes and Gilbert (15) set up the first balance sheet of ingested protein, fat, and carbohydrate as compared with the formation of these same substances in pigs, and demonstrated conclusively that triglycerides could be formed from carbohydrate This work has been amply confirmed by many types of investigation, including the use of isotopic labeling so that there is no question that neutral fat can be formed from carbohydrate The same conclusion has been drawn from experiments on micro organisms and on higher plants The increase of fat with simultaneous decrease in carbohydrate which occurs in the ripening of seeds has been observed in many species The yeast *Torulopsis lipofera*, grown in a glucose medium, forms fat at a rate of 4 to 11 per cent of its dry weight in 5 hours (15), far exceeding the rate of one per cent of body weight per day observed in abundantly nourished swine

Since the formation of fat from carbohydrate involves reduction it inexorably follows that some substance must be simultaneously oxidized Also, since fat formation involves energy storage, the stored energy must be supplied by an energy yielding process Both of these requirements are met by the oxidation of carbohydrate The biosynthesis of fat from carbohydrate has never been observed to proceed under anaerobic conditions Some anaerobic organisms can synthesize fat from alcohol or other non carbohydrate precursors Most micro organisms, including yeasts require oxygen for fat synthesis

In the animal body the synthesis of fat is not limited to any particular organ Evidence of lipid formation has been observed in liver, skin intestine, and brain (15) The lipid synthesis of the brain is most rapid at the time of myelinization and utilizes fatty acids locally synthesized rather than those supplied in the diet Adipose tissue has, in common with other tissues, the ability to form glycogen from glucose and to convert glucose to lipids

The pathway of fatty acid synthesis appears to be through two carbon fragments from acetic acid and alternatively pyruvic acid In liver slices the conversion of tagged acetic acid to fatty acids is demonstrable Such fatty acid synthesis is increased in the presence of pyruvic acid, with a still greater increase if insulin is also present (2) Elongation of fatty acid chains can occur by the addition of two carbon atoms at the carboxyl end The two carbon fragment can be derived from acetic acid as indicated by the use of labeled acetate (30) For the interrelations of two-carbon fragments (acetyl and carboxy methyl) in fatty acid synthesis see table 31

Synthesis of Sterols

Both plant and animal cells synthesize the cyclopentenophenanthrene ring which characterizes the sterols and steroids Animals are not capable of absorbing or utilizing the sterols which are formed by plants Vitamin D_2, which is irradiated ergosterol is absorbable and utilizable by animals, but it should be noted that the sterol ring is broken in the process of its formation from ergosterol, a plant sterol, by irradiation The parent plant sterol, ergosterol, is not absorbed

No single enzyme, nor any group of known enzymes is adequate to bring about the synthesis of the sterol nucleus Performance of this feat appears to require the entire and intact cell Furthermore it has been difficult to fix the identity of the substances from which the sterol ring is constructed in the cell, and such knowledge as we possess has come from the use of radioactive tracers *Acetic acid* is one such substance, as shown by the fact that labeled acetic acid was found to be incorporated in the cholesterol of the tissues of the experimental animals *Acetaldehyde* and *ethyl alcohol* are even more extensively incorporated into cholesterol than acetic acid, but are probably less important physiologically since they are not known to be produced in large amounts in normal metabolic processes, whereas acetic acid is always present in equilibrium with the carboxy methyl two carbon fragment (see table 31) The synthesis of cholesterol occurs in the liver, as can be demonstrated by the use of liver slices *in vitro* If cells are disorganized, as in a liver homogenate no cholesterol synthesis takes place The ability to synthesize cholesterol is not limited to the liver (24)

Probably from cholesterol other steroids necessary for continued life and health are formed within the body The only apparent exceptions are the D vitamins, which are not true steroids but steroid derivatives without the intact ring Vitamin D_2 is the result of the breakage of the ring, brought about by ultraviolet radiation of ergosterol a fungal sterol Vitamin D_3 is the result of a similar action upon 7-dehydrocholesterol, a typical animal sterol Vitamin D_3 is formed in the human body in adequate quantities by an entirely physiological process and without requiring the intake of any sterol precursor, provided enough sunlight or other source of ultra violet radiation strikes the skin In our boreal environment, this does not occur, therefore our children must be fed supplementary D vitamins, either D_2 from irradiated plant material or D_3 from animal sources

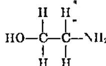
Abnormal Fat in Liver Cells

Ordinary yeast supplied with ethyl alcohol will convert it to fat, presumably by way of acetaldehyde and acetic acid Yeast supplied with glucose, which of course is converted by the yeast into ethyl alcohol, grows

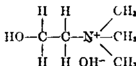
more vigorously but utilizes less of the sugar in fat production In many types of cells, fat formation is associated with senescence or with diminishing food supply *Damage to human liver cells is often indicated to the* examining pathologist by an accumulation of fatty acids and neutral fat within the cells These cells have as a normal function the production of the phospholipids which circulate in the blood and constitute the most available form of lipid for tissue utilization This function of hepatic cells is depressed, and abnormal intracellular fat deposits are produced, by certain poisons, including organic halides, carbon tetrachloride, and salts of heavy metals Failure of normal carbohydrate metabolism, as in diabetes, [illegible] infections, [illegible] do similar [illegible] ne tissue replacement of hepatic cells (cirrhosis), has long been recognized as associated with alcoholism Many ardent consumers of alcohol, however, show no evidence of liver cell abnormality Current clinical and biochemical thought considers these cases of "alcoholic fatty livers" as examples of a deficiency disease brought about by the limited food intake so often observed among alcoholics High carbohydrate diets have been found helpful in limiting the deposition of fat in liver cells in such cases, but high protein diets are even more effective The actual deficiency appears to be in *choline* and its dietary precursors, which include the amino acid methionine The function of methionine appears to be that of a donor of methyl groups, permitting the conversion of ethanolamine (formula V) to choline (formula VI) Ethanolamine itself may be formed by the reduction of glycine *or by the decarboxylation of serine* Thus, choline can be formed within the body, provided an adequate supply of methyl donors is available from dietary sources *Betaine* (formula VII) is another effective methyl donor, less significant than methionine in the normal diet, but useful in the treat*ment of patients with fatty livers*

Choline is designated as a *lipotropic substance*, which means that it is necessary for the normal production of phospholipids, which constitute the major portion of the indispensable lipids of cells In the absence of choline *neutral fat can not be converted into useful phospholipids* The rate of phospholipid synthesis in normal subjects has been measured (5) and found to be highly variable among different subjects, but relatively constant in the same subject over a period of months Administration of choline or methionine to the normal subject *does not increase the rate* of phospholipid formation, but such an increase can often be observed following the administration of large doses of choline or methionine to a patient with chronic hepatitis or cirrhosis It appears that much of the benefit derived from the high protein diet in such cases *was the result of the methionine* so supplied

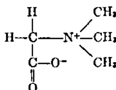
Pyridoxine is the type substance of the vitamin B_6 group which also includes pyridoxal and pyridoxamine These three substances are similar in biological effect and are lipotropic substances (23) in the sense that neutral fat accumulates in the livers of animals whose diets are chronically deficient in this vitamin group The oxidation of fatty acids appears to require pyridoxine or one of its derivatives which is not required in carbohydrate oxidation The vitamin B_6 group functions chiefly in the mammalian body as pyridoxal phosphate (see Chapter 19), which is necessary for the conversion of protein to fat probably through its function in the



V Ethanolamine



VI Choline



VII Betaine

decarboxylation and transamination of amino acids (see Chapter 14) The B_6 vitamins and the essential fatty acids both augment the lipotropic action of choline in rats each apparently having a specific function and each being indispensable Long continued pyridoxine deficiency in young monkeys has been followed by premature arteriosclerosis

Fatty livers can also be produced experimentally in pancreatectomized animals treated with insulin and they occur spontaneously in diabetics, particularly in juvenile diabetics Methionine choline and betaine can be used successfully in combating this type of fatty liver as well as the type caused by choline deficiency Inositol is lipotropic but only in a limited manner and in connection with diets low in fat (1) Certain pancreatic extracts (4) are also effective lipotropic agents but their mechanism m

is not understood They do not contain sufficient amounts of known lipotropic agents to explain their activity

A most extreme example of fatty degeneration of liver cells is seen in advanced cases of *kwashiorkor*, a disease of children caused by multiple dietary deficiencies, of which deficiency of protein seems to be the most significant In cases which have come to autopsy liver cells were found to consist of a single mass of fat which had pushed the nucleus aside and left only a thin shell [illegible]
some [illegible]
and p[illegible]
Africa, but has appeared in many localities where starchy foods, particularly maize manioc, or cassava, are the dietary staples (17), and there is lack of the proteins of high biological value supplied by such foods as milk, meat, and eggs

REFERENCES

1 Best, C H, *et al* Statistical evaluation of lipotropic action of inositol Biochem J, 48: 448–452, 1951
2 Bloch, K, and Kramer, W The effect of pyruvate and insulin on fatty acid synthesis *in vitro* J Biol Chem, 173: 811–812, 1948
3 Burton, K Higher fatty acid dehydrogenase of mammalian liver Nature 161 606, 1948
4 Canepa, J F, *et al* A study of lipotropic factors derived from the pancreas Am J Physiol, 156 387–395, 1949
5 Cornatzer, W [illegible] and Cater, D The effect of lipotropic factors on phospholip[illegible] active ph[illegible]
6 Dun[illegible]
7 Edsa[illegible] Uni versity Press, 1951
8 Fisher, P The role of the ketone bodies in the etiology of diabetic coma Am J M Sc, 221: 384–397, 1951
9 Friedemann, T E, *et al* An assessment of the value of the nitroprusside reaction for the determination of ketone bodies in urine Quart Bull Northwestern Univ M School, 20. 301–310, 1946
10 Gertler, M M, *et al* Diet, serum cholesterol, and coronary heart disease Circulation 2: 696–704, 1950
11 Geyer R P, and Waddell, W R Influence of carbohydrate on *in vivo* fat metabolism Federation Proc, 10: 383, 1951
12 Geyer, R P, *et al* Extrahepatic lipid oxidation in the rat Federation Proc 10, 188–189, 1951
13 Gofman, J W, *et al* Blood lipids and human atherosclerosis Circulation 2 161–178, 1950
14 Houssay, B A, *et al* *Human Physiology* New York, McGraw Hill, 1950
15 Kleinzeller, A Biosynthesis of lipids Advances Enzymol, 8 299–341, 1948
16 Krebs, H A The tricarboxylic acid cycle Harvey Lect, 44 165–199, 1948–49
17 Meiklejohn, A P, and Passmore, R Nutrition and nutritional disease Ann Rev Med, 2: 129–154, 1951

18 MORRISON L M Arterios [illegible]rosis J A M A 145 1232-1236 1 51
19 LOTTER V R AND HEIDELBERGER C Altern[illegible]e metabolic p[illegible]thways Physiol Rev 30 487-512 1950
20 PRICE T D AND RITTENBERG D The met[illegible]sm of acetone I Gross aspects of catabolism and exc[illegible]n J Biol Chem 185 449-459 1950
21 RENOLD A E AND MARBLE A Lipolytic act[illegible] of adipose t[illegible]ue in man and rat J Biol Chem 185 367 375 1950
22 SAKAMI W AND LAFAYE J M Formation [illegible]rmate and lab[illegible] methyl groups from acetone in the [illegible] rat J Biol Chem 187 369-378 1950
23 SHERMAN H Pyridoxi[illegible] [illegible] fat metabolis[illegible] Vitamins & H[illegible] 8 55-68 1950
24 SREBR[illegible] A et al Th[illegible] [illegible]hepatic synth[illegible] f cholester[illegible] J Biol Chem 182 629-631, 1950
25 THANNHAUSER S J [illegible] Diseases of [illegible] llular lipid m[illegible]olism Oxford Medicine New York Oxford University [illegible] 1949 vol [illegible] 2 Chapter XIII A
26 VERKADE P E et al R[illegible]arches on fat [illegible]lism XII The influence of carbohydrate on di[illegible] Biophys et B[illegible] Acta 2 [illegible] 1948
27 WEINH[illegible] S et al Th[illegible] effect of carbohy[illegible] n the oxidation of fatty acids [illegible] liver slices J B[illegible] Chem 181 489-4[illegible] 1949
28 WEINHOUSE S et al O[illegible]lation of isotop[illegible] [illegible]ic acid in an[illegible] tissues J Biol Chem 185 1[illegible] 1950
29 WERTHEIMER E AND S[illegible]ro B The p[illegible] [illegible]gy of adipose tissue Physiol Rev 28 451-464 1948
30 ZABIN I On the c[illegible] [illegible] f palmitic ac[illegible] stearic acid in animal tissues J Biol Chem 189 [illegible] 1951

is not understood They do not contain sufficient amounts of known lipotropic agents to explain their activity

A most extreme example of fatty degeneration of liver cells is seen in advanced cases of *kwashiorkor*, *a disease of children caused by multiple* dietary deficiencies of which deficiency of protein seems to be the most significant In cases which have come to autopsy liver cells were found to consist of a single mass of fat which had pushed the nucleus aside and left only a thin shell of *surrounding cytoplasm In cases which recover* some degree of connective tissue replacement of liver cells (cirrhosis) occurs and persists Kwashiorkor was identified and named in the Gold Coast of Africa but has appeared in many localities where starchy foods particularly maize manioc or cassava are the dietary staples (17) and there is lack of the proteins of high biological value supplied by such foods as milk meat and eggs

REFERENCES

1 BEST C H *et al* Statistical evaluation of lipotropic action of inositol Biochem J 48 448–452 1951

2 BLOCH K AND KRAMER W The effect of pyruvate and insulin on fatty acid synthesis *in vitro* J Biol Chem 173 811–812 1948

3 BURTON K Higher fatty acid dehydrogenase of mammalian liver Nature 161 606 1948

4 CANEPA J F *et al* A study of lipotropic factors derived from the pancreas Am J Physiol 156 387 395 1949

5 CORNATZER W E AND CAYER D *The effect of lipotropic factors* on phospholipide turnover in the plasma of normal persons as indicated by radioactive phosphorus J Clin Investigation 29 534–541 1950

6 DUNCAN G G *Diabetes Mellitus* Philadelphia W B Saunders 1951

7 EDSALL J T *Enzymes and Enzyme Systems* Cambridge Mass Harvard University Press 1951

8 FISHER P The role of the ketone bodies in the etiology of diabetic coma Am J M Sc 221 384 397 1951

9 Fi ... le reac ... estera

10 GERTLER M M *et al* Diet serum cholesterol and coronary ... sease Circulation 2 696–704 1950

11 GEYER R P AND WADDELL W R Influence of carbohydrate on *in vivo fat* metabolism Federation Proc 10 383 1951

12 GEYER R P *et al* Extrahepatic lipid oxidation in the rat Federation Proc 10 188–189 1951

13 GOFMAN J W *et al* Blood lipids and human atherosclerosis Circulation 2 161 178 1950

... York McGraw Hill 1950

... Enzymol 8 293-311 1948

... Lect 44 166-199 1948-49

17 MEIKLEJOHN A P AND PASSMORE R Nutrition and nutritional disease Ann Rev Med 2 129–154 1951

18 MORRISON L M Arteriosclerosis J A M A 145 1232 1236 1951
19 POTTER V R AND HEIDELBERGER C Alternative metabolic pathways Physiol Rev 30 487-512 1950
20 PRICE T D AND RITTENBERG D The metabolism of acetone I Gross aspects of catabolism and excretion J Biol Chem 185 449-459 1950
21 REYNOLD A E AND MARBLE A Lipolytic activity of adipose tissue in man and rat J Biol Chem 185 367 375 1950
22 SAKAMI W AND LAFAYE J M Formation of formate and labile methyl groups from acetone in the intact rat J Biol Chem 187 369-378 1950
23 SHERMAN H Pyridoxine and fat metabolism Vitamins & Hormones 8 55-68 1950
24 SRERE P A et al The extrahepatic synthesis of cholesterol J Biol Chem 182 629-634 1950
25 THANNHAUSER S J Lipidoses Diseases of the cellular lipid metabolism Oxford Medicine New York Oxford University Press 1949 vol 4 pt 2 Chapter VII A
26 VERKADE P E et al Researches on fat metabolism XII The influence of carbohydrate on diaciduria Biophys et Biochem Acta 2 38-56 1948
27 WEINHOUSE S et al The effect of carbohydrate on the oxidation of fatty acids by liver slices J Biol Chem 181 489-498 1949
28 WEINHOUSE S et al Oxidation of isotopic palmitic acid in animal tissues J Biol Chem 185 191 199 1950
29 WERTHEIMER E AND SHAPIRO B The physiology of adipose tissue Physiol Rev 28 451 464 1948
30 ZABIN I On the conversion of palmitic acid to stearic acid in animal tissues J Biol Chem 189 355-359 1951

CHAPTER 14

Proteins and Starvation

With the exception of the vitamins and certain essential fatty acids, the proteins are the only class of organic foodstuffs which are absolutely indispensable for animals One may live without dietary carbohydrate and probably the fat needs are confined to a few unsaturated fatty acids but without a certain minimal amount of protein, the human body can not continue to live and operate normally This is not surprising since life is essentially based on proteins

We have already seen how proteins are broken down in the course of digestion and noted how only a minute amount is absorbed in the form of intact protein molecules which do not seem to confer any particular benefit when absorbed in this form, and may do mischief to the allergic individual It is not the intact proteins which are essential, but the amino acids to which they are hydrolyzed during metabolism

Not even all the amino acids are essential in our diet, we may classify the amino acids which occur naturally in proteins into two groups *essential* and *non-essential* The essential amino acids, or amino acid food factors, have been listed and their nutritional significance discussed in Chapter 10 By means of nitrogen balance experiments in young men, Rose (24) has determined the minimal amount of each of the essential amino acids required to keep the human subject in nitrogen equilibrium where there is an adequate total intake of protein nitrogen to synthesize the non-essential amino acids These values are shown in table 32 The recommended dietary allowance is double the minimum It is necessary not only to have enough protein, therefore, but also enough of proteins which together contain enough of the essential amino acids It has been found that animal proteins are, in this respect, more suitable for human nutrition than are the proteins in plants The protein of maize, zein, is deficient in tryptophane and lysine, and if taken as the sole protein of a diet, is not adequate to establish nitrogen equilibrium Gelatin a derived protein, is also deficient in tryptophane and in phenylalanine, and is therefore another protein of poor biological value Zein and gelatin are two of the poorest proteins we know, not sufficient in themselves to supply several

of the amino acids needed in nutrition. Although plant proteins are less effective nutritionally than the animal proteins, it is perfectly possible for human beings to live on a purely vegetable diet, and many persons do so.

Not only must the essential amino acids be supplied in adequate amounts, but they must be supplied approximately simultaneously. Geiger (9) fed rats on an amino acid mixture which supplied all the amino acids except one. The missing amino acid was in some experiments tryptophane, in others methionine or lysine. Even if the missing essential amino acid was consistently fed several hours after the incomplete mixture, growth was not maintained. In later experiments with pairs of proteins which together supplied the essential amino acids, but failed to do so separately, satisfactory growth was observed when the complementary pairs of proteins

TABLE 42

Minimal requirements of essential amino acids by man in grams per day

Leucine	1.10
Methionine	1.10
Phenylalanine	1.10
Valine	0.80
Lysine	0.80
Isoleucine	0.70
Threonine	0.50
Tryptophane	0.25

were fed together, but not when they were fed alternately with a lapse of time between (10).

GENERAL METABOLISM OF AMINO ACIDS

The amino acids which are liberated by digestive hydrolysis of proteins are taken up by the portal vein, and are distributed in the general circulation. Following a protein meal, the amino nitrogen of the blood increases by 2 to 6 mgm. over its fasting level of 4 to 6 mgm. per 100 ml., and remains at an elevated level for about 6 hours.

The utilization of amino acids by the body may be either *structural* in the formation of body proteins, or *energic* by conversion into nitrogen-free intermediates, which can be metabolized by the same mechanisms which make available the energy of fat and carbohydrate. The synthesis of protein from amino acids may occur conceivably by simple reversal of proteolysis catalyzed by proteinase, the direction being determined by mass action and the energy supplied by high energy phosphate bonds. Experimental attempts at *in vitro* synthesis of proteins by reverse enzyme action has resulted only in the production of *plasteins*, which are insoluble

polypeptides. The production of peptide linkages, e.g., the formation of *p*-aminohippuric acid from *p*-aminobenzoic acid and glycine, by the enzyme systems present in kidney or liver slices requires aerobic conditions and the presence of ATP and cytochrome (5), hence is not a simple reversal of hydrolysis. In general, the incorporation of tagged amino acids experimentally into mammalian tissue proteins, e.g., in liver slices requires aerobic conditions. It has already been indicated in an earlier chapter that the nucleic acids may have a specific function in protein synthesis. Caspersson (see Chapter 7) has observed increased PNA content of cells during active protein production. Depending on their type of energic utilization amino acids may be divided into two main classes, according to whether

TABLE 33

Fates of amino acids administered to a diabetic or phlorizinized animal

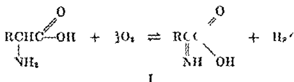
GLUCOGENIC	KETOGENIC	FATE UNKNOWN
Glycine	Leucine	Tryptophane
Alanine	Isoleucine	Lysine
Serine	Phenylalanine	
Threonine	Tyrosine	
Cysteine		
Histidine		
Methionine		
Valine		
Aspartic acid		
Glutamic acid		
Arginine		
Ornithine		
Proline		

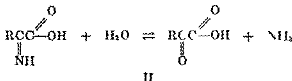
they may be converted in the body into carbohydrates or into ketone bodies. There are two amino acids, both essential, which are not known to give rise to either glucose or ketone bodies. Their fate in the human body is unknown. This gives us the three categories of table 33.

The decision which establishes a particular amino acid as glucogenic or ketogenic has usually been based upon experiments involving the feeding of the amino acid to an animal rendered diabetic by pancreatectomy or glycosuric by the use of the glucoside phlorizin. In such an animal the feeding of a glycogenic amino acid leads to an increased output of urinary glucose, the feeding of a ketogenic amino acid leads to increased output of ketone bodies. The ratio of glucose (dextrose) to nitrogen in the urine of a phlorizinized animal (the D/N ratio) is 3.65 when the animal is fed a diet consisting entirely of protein. Since protein contains approximately 16 grams of nitrogen per 100 grams protein, the urinary glucose supplied

by 100 grams of protein would be 3.65 × 16 or approximately 58 grams of glucose. This is the basis of the customarily applied dietetic rule that 58 per cent of protein is convertible to glucose in the body.

Deamination. One of the first things that happens to amino acids in their energic metabolism is apparently the removal of the amino group. This is accomplished in two steps by the catalytic action of *amino acid oxidases*. The first step is a catalyzed aerobic dehydrogenation forming an imino acid (formula I). The second step is a spontaneous hydrolysis of the imino acid to ammonia and a keto acid (formula II). There are two amino acid oxidases with group specificity present in the body, one for the D series and one for the L series of amino acids. It is interesting that it has been much easier to demonstrate the enzyme which acts on the unnatural D series. The enzyme for deaminating the D series actually seems more con-

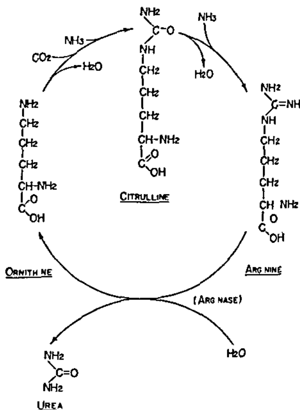




centrated. Both oxidases are found in liver and kidney, and both are flavoprotein enzymes. The natural, or L-amino acid oxidase, does not function with glycine or with the diamino dicarboxylic or beta hydroxy amino acids. Special mechanisms, to be explained later, are required for the deamination of these substances. The L-amino acid oxidase is a flavoprotein with a prosthetic group of riboflavin phosphate. The D-amino acid oxidase is non-specific and consists of FAD joined to protein.

Urea formation. The ammonia which is split off from amino acids in the process of deamination is ultimately converted to urea and eliminated in the urine in that form. Urea is one of the simplest and best known of all biochemical substances. Wohler in 1828 accidentally synthesized urea from ammonium cyanate, NH_4NCO. This event, reputedly occurring on the back of a cook stove, was the beginning of organic chemistry as we know it today and of biochemistry. We customarily regard urea as it is shown in the formula, the diamide of carbonic acid, or the amide of carbamic acid, or *carbamide*. In theory, we can derive urea by the addition of two mole-

cules of ammonia to one of carbonic acid, with the loss of two molecules of water This reaction will not proceed uncatalyzed *in vitro* and is accomp plished in the body in a very roundabout manner (formula III) Let us start in the eastern portion of the diagram The amino acid *arginine* is the specific substrate for an enzyme *arginase*, which is present in mam malian livers, but not in those of birds snakes or lizards This is significant

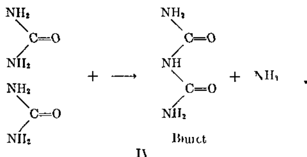


III The Krebs and Henseleit Urea Cycle

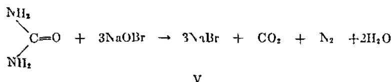
because mammals excrete most of their waste nitrogen in the form of urea whereas the other animals mentioned excrete theirs chiefly as uric acid Catalyzed by arginase, arginine is hydrolyzed to *ornithine* and *urea* The ornithine so formed adds CO and NH_3 to form *citrulline*, which in turn adds NH_3 to form arginine Note that in these two steps the components of urea are added and that in each of the steps a molecule of water is given off Arginine is now ready in the presence of arginase to liberate urea and be reconverted to ornithine to start the cycle again The presence and function of arginase in the liver had been long known before 1932, when

Krebs and Henseleit clarified the cyclic nature of this process and demonstrated that it was quantitatively important in urea formation In the diagram the processes by which urea is formed are represented as proceeding in one direction This is done only for the sake of simplicity since all these reactions are probably physiologically reversible Some certainly are since urea tagged with radioactive carbon may be injected into animals and more than 20 per cent of the carbon will be eliminated as CO_2 in the expired air the remainder appearing as urea in the urine

Urea is a colorless odorless solid stable in air with a melting point of 133°C It is soluble in water and its water solution has a cool saline taste Urea is also soluble in alcohol but not in ether or chloroform Acetone and



IV

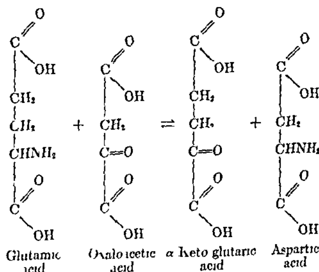


V

amyl alcohol are suitable solvents for its recrystallization Urea forms a relatively insoluble oxalate and nitrate Moist heat above 140°C splits urea to ammonia and carbonic acid by addition of water The same effect can be obtained at ordinary temperatures by the use of the enzyme urease (from jack beans or soybeans) or by enzymes of the urea-splitting bacteria Heating urea dry causes loss of ammonia and the formation of biuret (formula IV) With two molecules of xanthydrol urea forms insoluble dixanthylurea which has exactly seven times the molecular weight of urea and is a suitable substance for gravimetric analysis of urea The reaction of urea with hypobromite (formula V) has been made the basis of gasometric analysis Colorimetric analysis can be accomplished by Nesslerization of the ammonia liberated by urease action or by heating in the autoclave

The experiments of Mann Bollman and Magath have proven quite

conclusively that urea is formed in the liver Their work involving extirpation of the liver has shown that, in dogs at least, no significant amount of urea is formed elsewhere They observed the following results after hepatectomy (a) fall in blood sugar, (b) fall in blood urea, with disappearance of urea from the urine within 36 hours (c) no conversion of injected amino acids or ammonium compounds into urea, and (d) increase of blood ammonia These results were foreshadowed in the early 1890's by the work of Pavlov, Nencki, and others who established in experimental animals an *Eck fistula* which connected the portal vein to the inferior vena cava



VI

thereby short circuiting the portal circulation through the liver Such animals showed diminished urea output in the urine

Transamination Under the influence of enzymes called *transaminases* with pyridoxal phosphate (see page 692) as coenzyme, amino groups can be transferred from one compound to another in the body Most commonly the nitrogen donor is an amino acid, and *glutamic acid* participates in the most rapid known transamination reaction (formula VI) In a similar manner alpha amino acids can react with other alpha keto acids For instance if glutamic acid and pyruvic acid are added together to chopped liver or muscle tissue, the alpha amino group of the glutamic acid is partly transferred to pyruvic acid so that alpha ketoglutaric acid and alanine are formed This process approaches an equilibrium which can be reached from both sides Thus if we add alpha ketoglutaric acid and alanine to such a tissue preparation, glutamic acid and pyruvic acid are formed until

the same equilibrium is reached Since this mechanism of transamination exists, any of the amino acid food factors can be replaced experimentally in the diet by the structurally corresponding keto acid (i e phenylalanine by phenyl pyruvic acid) and no deficiency will occur provided adequate nitrogen is supplied, as by non-essential amino acids, to provide nitrogen donors for transamination *Lysine* and *threonine* are exceptions Their keto acids can not accept nitrogen by transamination (8)

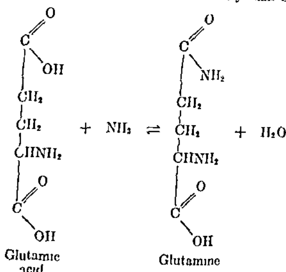
This transamination of amino acids seems to be going on constantly, and Schoenheimer and his colleagues (27) were able to demonstrate that no amino acid retains its amino group for long inside of the body Instead, the whole pool of amino acids in the body is in a constant process of change

The dynamic state of the protein constituents of the body was first proved by Schoenheimer and his group, using N^{15} to label the introduced amino acids They found that all the complex molecules of the body, including proteins and their components amino acids and nucleic acids, were constantly involved in rapid chemical reactions Fats and carbohydrates are likewise in a state of flux Ester peptide, and other linkages open, fragments are liberated and merge with those derived from other large molecules, and these form a metabolic pool of components which can no longer be distinguished in regard to origin These liberated molecules are again subject to numerous processes, fatty acids are dehydrogenated, hydrogenated, degraded, or elongated, and in general continually converted While some individual molecules of these fatty acids are completely degraded, other individuals of the same chemical species are steadily being formed from other substances—notably, carbohydrate

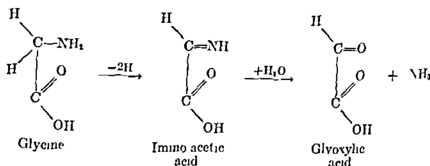
Similar reactions are found to occur among the split products of proteins The free amino acids are deaminated and the liberated nitrogen transferred to other previously deaminated molecules to form new amino acids What had seemed to be a very stable situation turned out to be another example of the dynamic equilibrium which we have come to associate with life

If growing rats are fed only the essential amino acids, and only in the amounts necessary to give maximum growth when the other non-essential amino acids are abundantly supplied, growth is very slow The lack of nitrogen is the limiting factor in this situation In place of the non-essential amino acids, urea, or ammonium salts, such as diammonium citrate, may serve as a source of nitrogen (25) Such utilization of ammonium salts must take place promptly since ammonia is distinctly toxic Blood ammonia concentrations of the order of 1:20,000 are lethal to rabbits Ammonia can be stored, chiefly in the liver, in the form of the amide of glutamic acid, glutamine (formula VII), forming a reserve of non toxic but chemically available ammonia for the synthesis of amino acids The carbon for the

synthesis of non-essential amino acids may be supplied by acetate, as shown by tracer experiments (11), much more effectively than by CO_2



VII



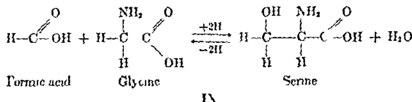
VIII

Metabolism of Individual Amino Acids

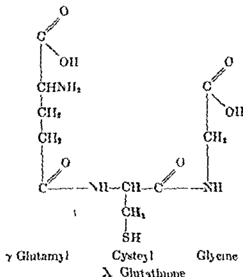
Glycine is a non-essential glycogenic acid and is the simplest naturally occurring amino acid, in fact the simplest possible alpha amino acid On deamination by its specific flavoprotein enzyme, *glycine oxidase*, glycine gives rise to glyoxylic acid (formula VIII) From the glyoxylic acid so formed, one possible product is formic acid Formic acid so derived, or derived from the methyl groups of methionine or choline, may react with glycine to form serine (28) (formula IX)

Glycine is found as two methylated derivatives, sarcosine, a monomethyl

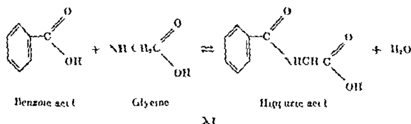
glycine and glycylbetaine It is also found in the peculiar tripeptide called glutathione (formula X)



IX



X Glutathione



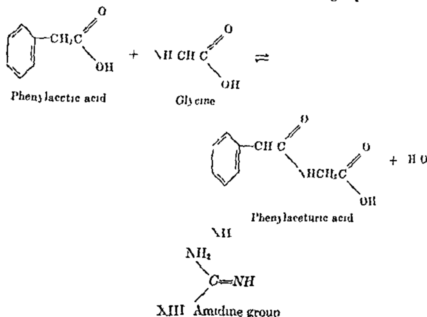
XI

Glycine plays an important part in the renal elimination of aromatic acids such as benzoic and phenylacetic acid These are excreted as conjugates of which formulas XI and XII are examples Of these, hippuric acid the conjugation product of glycine with benzoic acid has been most extensively studied Regardless of the glycine intake, the human body can,

if benzo . . . d . f . .

are at least

Tracer experiments with glycine tagged with isotopic nitrogen demonstrate that the nitrogen of glycine appears in heme, and as the nitrogen of *position 7 in the purine ring If the carboxyl carbon of glycine is tagged, it will appear in position 4 of the purine ring* The carboxyl carbon of glycine is not utilized in porphyrin synthesis, but the *methylene or alpha-carbon* (the "amino" carbon) of glycine is introduced into eight positions in the



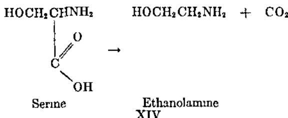
porphyrin ring of heme A2, B2, C2, D2 and the four methene bridge carbons (37) Glycine in itself is a major component of collagen (see page 128) and of glycocholic acid (see page 123) Glycine adds the amidine group (formula XIII) from arginine to form glycocyamine or guanidinoacetic acid, which is a physiological precursor of creatine (see page 553)

Alanine is non essential and glycogenic On deamination or transamination it yields pyruvic acid, which is known to be on the direct line of the breakdown and synthesis of glucose and glycogen Only a small portion of the carbon of tagged alanine actually appears in glycogen, but ingestion of alanine by a diabetic animal gives a quantitative increase in glucose output Here, as with other glycogenic amino acids except glycine, it is assumed that the deaminated derivatives in this case pyruvic acid, are preferentially oxidized, thereby sparing glucose or other glycogenic metabolites

Serine is non-essential and glycogenic and is not as common as some of the other amino acids, and not so much is known about its metabolism There is a specific enzyme present in the liver which deaminates serine to yield pyruvic acid Serine is also in part converted by decarboxylation to *ethanolamine*, which is involved in phospholipid synthesis (formula XIV)

Threonine is essential and glycogenic Like serine it is a hydroxy amino acid but it is not understood why the longer hydroxyamino acid should be essential when the shorter one is not The metabolism of threonine is not known in any detail It does not participate in transamination

Cysteine and cystine are non-essential and glycogenic The two amino acids together form a reversible oxidation reduction system, as will be readily appreciated from an inspection of the formulas which show that cystine represents two molecules of cysteine from which we have removed two atoms of hydrogen Glutathione in its reduced form contains cysteine, in its oxidized form, cystine (formula XV) Cysteine activates folic acid con

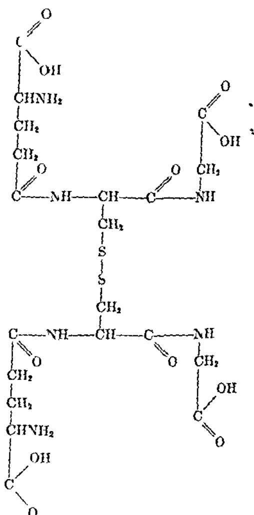


XIV

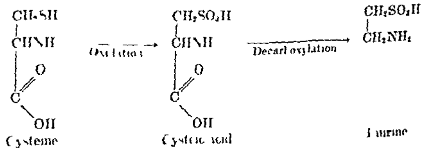
jugase (see page 697) in the kidney of the hog by transfer of its SH group to the inactive enzyme precursor (12) Many other enzymes depend upon free SH groups for their activity Cysteine is also the parent substance of taurine, beta-aminoethylsulfonic acid, a compound which in man is found conjugated in the bile salts (page 123) Taurine is formed by the enzymatic decarboxylation of cysteic acid which is derived, as shown in formula XVI, by the oxidation of cysteine

before, has a ready mechanism for the conversion of this to a non toxic compound shown in the formula, parabromophenylmercapturic acid (formula XVII) Other mercapturic acids are known

Cystinuria is a metabolic anomaly in which cystine is excreted in the urine in large amounts, often with the formation of bladder stones The excretion

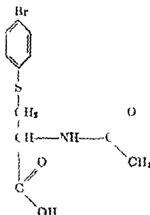


XX Glutathione (oxidized form)



of cystine in cystinurics continues during fasting and curiously enough is not increased by the feeding of cystine It is increased by the feeding of cysteine or of methionine but less so if the subject is on a high protein diet Methionine tagged with radioactive S appears in the urine of cystinurics as cystine A more serious anomaly of cystine metabolism is cystine storage disease in which there is renal damage and bony abnormalities similar to rickets (17)

Injections of cysteine have a protective effect in rats against the effects of x radiation Cysteine protected rats who have received 800 r total body x irradiation have more neutrophils in the blood and more developing myeloid cells in the bone marrow, as compared with saline injected controls



XVII p Bromophenylmercapturic acid

who received the same irradiation (26) X rays and other types of ionizing radiation oxidize SH groups in aqueous solution (1)

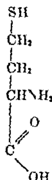
Methionine is an essential amino acid and is glucogenic It is possible that its main function is as a biological methylating agent (see page 528) It is known to complete the synthesis of creatine by transferring a methyl group to glycocyamine It may be the methylating agent involved in the detoxication of pyridine and certain pyridine derivatives including nicotinic acid Methionine is the chief source of methyl groups in the synthesis of choline in the body and hence has a *lipotropic* activity (see page 528) Methionine functioning in this manner has been found useful in the treatment of the hepatic cirrhoses developed in rats by a low protein diet, and in fatty livers and portal cirrhoses in the human

After being demethylated methionine is converted into homocysteine (formula XVIII) the sulfhydryl group of which can be transferred to serine, resulting in the synthesis of cysteine As an essential amino acid, methionine

can not be replaced by homocysteine, but is replaceable by homocysteine plus choline or betaine The betaine adds a methyl group to homocysteine yielding methionine Choline does not transfer a methyl group directly but is first converted to betaine (21)

The ethyl analogue of choline *triethylcholine*, or the ethyl analogue of methionine *ethionine* will inhibit growth in rats probably by forming ethyl analogues (29) of essential metabolites which normally carry methyl groups In mice the presence of triethylcholine will diminish the formation of acetylcholine (14) These are examples of metabolite antagonism (see page 736) in which a compound structurally similar to a normal metabolic product blocks the mechanism by which it is normally utilized

Valine is essential and glycogenic but was at one time believed to be ketogenic For it to be converted into glycogen one of the methyl groups of



XVIII Homocysteine

valine after its deamination must be removed, but the mechanism by which this is accomplished is still unknown, as is the specific metabolic function which makes valine essential

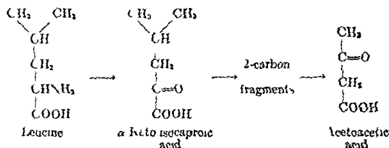
Leucine is essential and ketogenic Its conversion into ketone bodies can be explained by the hypothetical series of reactions shown in formula XIX

Isoleucine which is an isomer of leucine is essential and ketogenic and might be converted into acetoacetic acid by a mechanism similar to those postulated for leucine

Aspartic acid is non-essential and glycogenic On transamination it yields oxaloacetic acid which is an intermediate in carbohydrate metabolism In this way aspartic acid can enter the Krebs cycle (see page 496) Also aspartic acid can be formed by the transfer of amino groups to oxaloacetic acid Aspartic acid *is not deaminized* by L amino acid oxidase Under the influence of the enzyme asparaginase aspartic acid can react with ammonia to form *asparagine* This is also non-essential and glycogenic Like glutamine asparagine may function as a storage place for amino groups

Aspartic acid in proteins occurs chiefly as asparagine and is converted to aspartic acid by hydrolysis

Glutamic acid is one carbon longer than aspartic acid and is also non essential and glycogenic It yields alpha ketoglutaric acid on deamination This product is oxidatively decarboxylated in the usual way and produces succinic acid The role of succinic acid in metabolism has already been discussed Glutamic acid has an important part in the process of trans amination and is a constituent of glutathione It occurs in proteins chiefly as its amide *glutamine* which has been discussed in relation to amino group storage and urea formation The glutamine of the blood is the chief source of urinary ammonia (see page 556) Glutamic acid and glutamine are present in high concentrations in brain as compared to other tissues In the presence of glucose brain slices will take up glutamic acid even when there is already



XIX

more glutamic acid within the cell than outside Weil Malherbe (35) has documented the concept that a major function of glutamic acid in the brain is the removal of ammonia which is particularly toxic to neural tissue by the formation of glutamine

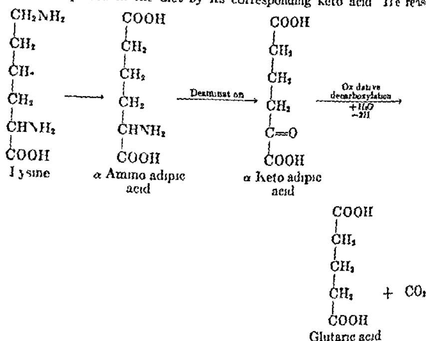
Arginine is non-essential and glycogenic It can lose part of its guanidine group under the influence of the enzyme arginase to yield urea and *ornithine* which is also glycogenic Arginine is the source of the amidine group of glycocyamine and creatine

Citrulline is non-essential and glycogenic As its name suggests it was isolated from the juice of the watermelon It is not known to enter into the composition of natural proteins, but is is an intermediate step between ornithine and arginine in the ornithine cycle of urea formation

Ornithine is non-essential and glycogenic but it has not been isolated from products of the hydrolysis of proteins It arises from the action of arginase on arginine and participates in the ornithine cycle (page 536) It may be one of the amino acids which is not deaminated in the body,

As the name suggests the amino acid ornithine has a special function in birds where it aids in the detoxication of aromatic acids such as benzoic acid

Lysine is an essential amino acid and is neither glycogenic nor ketogenic It has the longest aliphatic carbon chain known to occur in proteins Little seems to be known about the function of lysine in the human body and it is not understood why it is an essential amino acid Once deaminated, it can not accept an NH_2 group by transamination in the body, hence can not be replaced in the diet by its corresponding keto acid The reason



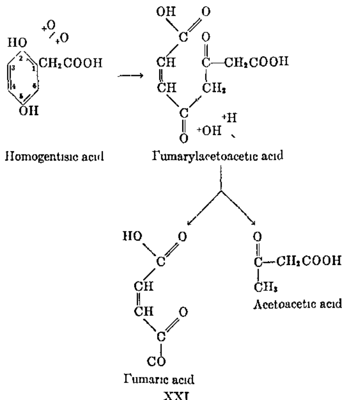
XX

for this is that lysine is metabolically altered before transamination The epsilon-amino group is subjected to oxidative deamination forming alpha amino adipic acid which can then be deaminated in the usual manner forming alpha keto adipic acid This keto acid by an oxidative decarboxylation is converted to glutaric acid (formula XX)

Under the influence of bacterial enzymes lysine is decarboxylated and yields the diamine *cadaverine*, which like the similar *putrescine* derived from ornithine is toxic but can be oxidatively detoxicated by diamine oxidase

Phenylalanine and *tyrosine* may be considered together Of the two phenylalanine is essential in the dietary sense Tyrosine has more known functions than has phenylalanine but in the body tyrosine can be formed

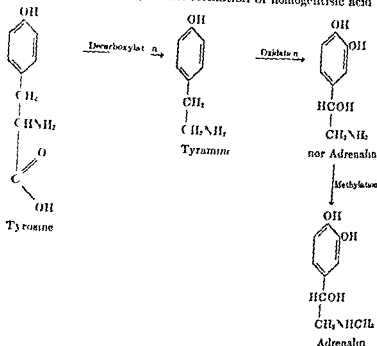
from phenylalanine, and the reverse does not occur Both phenylalanine and tyrosine are ketogenic, forming acetoacetic acid in rat liver slices (16) If phenylalanine is used it is converted in the liver slice to tyrosine By oxidative deamination and decarboxylation, tyrosine leaves a residue of *p* hydroxyphenylacetic acid (a normal urinary constituent in small amounts, see page 658) By a rearrangement and further oxidation, the intermediate



XXI

steps of which are not known, *homogentisic acid* is formed This substance occurs in the urine only in those individuals who can not carry through the next step in the metabolic process the oxidative breaking of the benzene ring Ravdin and Crandall (23) have demonstrated that the ring must open between carbons 1 and 2 forming by oxidation 4 fumarylacetoacetic acid This intermediate hydrolyzes into acetoacetic acid and fumaric acid (formula XXI) In a congenital metabolic anomaly, *alkaptonuria*, ring breakage does not occur, and such individuals excrete homogentisic acid in the urine, which darkens on exposure to air (see page 658) Homogentisic

acid is also excreted in the urine of scorbutic guinea pigs, along with hydroxyphenyl pyruvic and p hydroxyphenyllactic acids, which latter ac also occur in the urine of premature human infants deficient in vitamin Alkaptonuria has been a useful situation for the experimental study tyrosine metabolism in the human The excretion of homogentisic ac persists during fasting in alkaptonurics, but is increased by the feeding tyrosine of phenylalanine and of any substance derived from tyrosine its normal metabolism up to the formation of homogentisic acid There



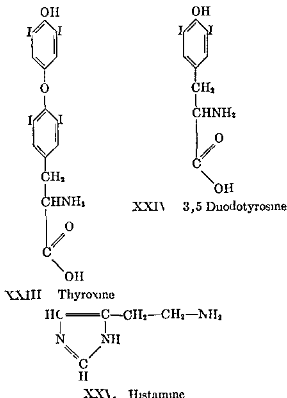
XXII

no interference in the structural utilization of tyrosine, or in the formation of other tyrosine derivatives about to be mentioned In association with alkaptonuria and sometimes independently, may be observed *ochronosis*, which is the pigmentation of cartilage and sclera with homogentisic acid oxidation products *Tyrosinosis* is the urinary excretion of unusual amounts of unchanged tyrosine and its deamination products (20)

The common black animal pigment melanin is formed by the oxidation and condensation of tyrosine initiated by the enzyme tyrosinase In the inherited peculiarity known as albinism this enzyme is lacking and the melanin pigments are completely absent from the skin, hair, eyes, and other usual situations The mechanism by which *adrenalin* may be formed from tyrosine is shown in formula XXII *Thyroxine* (formula XXIII) and

diiodotyrosine (formula XXIV) are iodinated derivatives of tyrosine, and are formed by the iodination of tyrosine containing proteins *in vivo* and *in vitro*

Histidine is non essential and slowly glycogenic It occurs in combination with the unusual compound beta alanine in the form of the dipeptide *carnosine* which is present in the muscles of most vertebrates, and also



XXIII Thyroxine

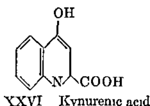
XXIV 3,5 Diiodotyrosine

XXV. Histamine

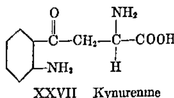
in *anserine*, which is a methyl carnosine Another histidine compound of possible physiological significance is the *ergothioneine* of the red blood cells The liver enzyme *histidase* converts histidine into glutamic acid, with liberation of ammonia The glutamic acid so formed may account for the moderate glycogenic property of histidine The enzyme histidine decarboxylase, which is present in traces in the liver and kidney, is capable of converting histidine to the very toxic amine, *histamine* (formula XXV) Histamine seems to be actively concerned with the manifestations of various types of allergic reactions (4)

Since there is no evidence that the imidazole ring of histidine can be synthesized in the body it is disturbing that histidine is not an essential amino acid Nevertheless adult men and rats can be kept in nitrogen balance without histidine and no strictly necessary function other than as a structural component of proteins has been found for histidine or the histidine containing compounds of the body Of the small amounts of free amino acids ordinarily found in human urine histidine is present in greatest amount This excretion of histidine is greatly increased in pregnancy along with a general increase in amino acid excretion This fact has been the basis of proposed tests for pregnancy but these have not been as sensitive and specific as those pregnancy tests based upon the excretion of hormones (see Chapter 6) Growing rats require histidine probably for protein synthesis It is probable that growing children require it although this has not been experimentally demonstrated in a clear cut fashion

Tryptophane is essential but is not demonstrably ketogenic or glycogenic



XXVI Kynurenic acid



XXVII Kynurenine

If it is given to dogs or rabbits in fairly large doses it produces kynurenic acid (formula XXVI) Kynurenic acid does not occur in human urine From an intermediate product kynurenine (formula XXVII) animals probably including man can form *nicotinic acid* This member of the vitamin B complex can therefore be synthesized from tryptophane in amounts sufficient to meet part of the physiological requirement Dietary deficiency of tryptophane in rats leads to a specific decrease in certain enzyme activities in the liver (36) The oxygen uptake of homogenates from the livers of the deficient rats was decreased as were the specific activities of xanthine dehydrogenase and of succinic acid dehydrogenase

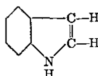
Degradation products of tryptophane by intestinal bacterial action are indole (formula XXVIII) and skatole (formula XXIX) which are said to be largely responsible for the odor characteristic of the feces If they are absorbed they are detoxicated by conjugation with sulfuric acid and excreted in the urine in the form of the so called ethereal sulfates

Proline is not strictly an amino acid but an imino acid It is non-essential and is glycogenic probably by its conversion to glutamic acid which has been demonstrated (32) Proline is also reversibly convertible to ornithine and arginine

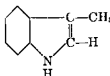
Hydroxyproline is non-essential and does not occur in many proteins It is not, however, an artifact of isolation (30) Hydroxyproline of the diet is not significantly incorporated into body proteins Such hydroxyproline as occurs in body proteins is formed by oxidation of proline, presumably after it is combined in peptide groups

Creatine is an amino acid but not of the usual alpha amino acid type, and it does not occur in protein It does not consistently appear in human urine, but is slowly converted under physiological conditions into its internal anhydride, *creatinine* which is the normal excretory product Its origin from creatine has been verified by feeding creatine labeled with heavy nitrogen

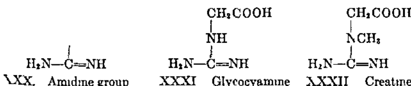
Creatine is formed physiologically by the interaction of several of the amino acids already discussed In kidney slices (3), the amidine radical



XXVIII Indole



XXIX Skatole

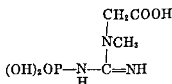


XXX Amidine group XXXI Glycocyamine XXXII Creatine

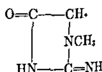
(formula XXX) of arginine is transferred to glycine, forming guanidino acetic acid or *glycocyamine* (formula XXXI) Glycocyamine is methylated in the liver, forming *creatine* (formula XXXII), which is methyl guanidino acetic acid The methylating agent is probably methionine When methionine loses its methyl group, it becomes homocysteine (see page 546) The methylation of glycocyamine to form creatine requires ATP and aerobic conditions

Creatine may be considered as a methylated amino acid, the non methylated parent substance being glycocyamine Glycocyamine, in amounts of about 20 mgm per day, is a normal urinary constituent (2) Its excretion in myasthenia gravis is not increased (34), as has been found to be the case with creatine *Certain foreign substances are methylated in the body before* excretion—pyridine, naphthalene (in part), and tellurium salts, which last are excreted chiefly as methyl telluride Many potent drugs, such as choline, muscarine, and adrenaline, are methylated compounds Ergothioneine, a reducing component of red blood discs, is a methylated thiohistidine Cre

atine is part of the muscle machinery, and as such was discussed in Chapter 3 In skeletal muscle the concentration of creatine is about 0 5 per cent In the resting muscle the creatine is almost entirely in the form of phos phocreatine (formula XXXIII), which splits during muscular activity, giving its high energy phosphate bond to ADP, and leaving free creatine The concentration of creatine is high in liver, kidney, and testicle, as well as in cardiac and skeletal muscle, but its function in non muscular tissue has not been clearly established Presumably, it acts as a carrier of high energy phosphate bonds there also Creatine is present in normal male blood serum in amounts between 0 17 and 0 58 mgm per 100 ml, in female blood serum between 0 35 and 0 93 mgm (33) Creatine is normally absent from the urine of healthy adult males, but is present in the urine of women when the serum level is 0 58 mgm per 100 ml, or over It is frequently found in the urine of normal children In hyperthyroidism, in states involving undernutrition, and very typically in diseases of the muscles, abnormal amounts of creatine appear in the urine



XXXIII Phosphocreatine



XXXIV Creatinine

Creatine given to a human subject by feeding or injection can not be entirely traced In the male, there is a slight transitory rise in the serum creatine level, with little or no creatinuria In the female the serum level rises higher and stays elevated for a longer time, and creatinuria occurs In both sexes some storage of creatine in muscle and viscera can be demon strated, but not enough to account for the amount given In myopathic patients, on the other hand, injected creatine is almost quantitatively excreted in the urine The feeding of creatine precursors, such as glycine arginine, or glycocyamine, will cause little change in creatine output by normal subjects, but will cause increases in the myopathic patient There is no evidence of any defect in transamidination or transmethylation in myasthenics (34)

Creatinine (formula XXXIV) is the cyclic anhydride of creatine, and is present in normal blood serum in concentrations from 0 9 to 1 65 mgm per 100 ml (33) Increases in blood creatinine occur only in serious renal in sufficiency The urinary excretion of creatinine is reasonably constant in the individual from day to day, and among individuals varies from 1 to 2 grams, with a very rough proportionality to body weight The urinary output is independent of protein intake or creatine intake Injected creatinine is

excreted in the urine to the extent of 75 to 96 per cent of the amount injected The rest disappears, possibly by diffusion into the intestinal tract where it is utilized by bacteria The creatinine of the urine has been judged to be derived from tissue creatine on the basis of similar isotopic ratios in tissue creatine and urine creatinine in animals given tagged creatine The reverse conversion creatinine to creatine, has not been demonstrated by isotopic methods It is quite possible that the steady output of urinary creatinine is the result of the spontaneous hydrolysis of phosphocreatine Lundquist (18) observed that phosphocreatine hydrolyzed at 38°C in saturated picric acid solution yields about 10 per cent of its weight as creatinine

Minimal Requirement of Protein

There is no doubt that a large part of the population of the world is under nourished and a certain part of it is constantly on the verge of starvation During prolonged fasting the excreted nitrogen can fall below 3 grams N per day corresponding to less than 20 grams of protein, but it has never been proved that 20 grams of protein is sufficient for a maintenance diet Many attempts have been made to establish a figure for the minimal human requirement of protein and this work has been summarized by Keys *et al* (15) No definite statement is justified other than that the requirement is higher than 20 grams and lower than the 1 gram per kgm of body weight which is recommended by the National Research Council Inherent in any statement of protein requirements is the question of the *quality* of the proteins used No amount of an incomplete protein, such as zein or gelatin, would constitute an adequate intake All recommendations imply the use of a diet including enough complete proteins to meet the needs for essential amino acid food factors (see page 533)

Protein Deficiency

The prolonged use of a diet inadequate in protein leads inevitably to growth failure in children and to loss of body protein in adults Actual loss of weight may not occur if the diet is calorically adequate or excessive, and fat may be stored as adipose tissue even in protein-deficient states Nutritional edema (see page 570) may mask losses of true body weight Chemical studies of severely underproteinized persons will show low levels of hemoglobin and of plasma protein, although these findings may be minimized by decreased plasma volume

Protein deficiency may occur not only from inadequate protein in the diet, or use of proteins lacking essential amino acids, but also from pathological states affecting the digestion, absorption, or utilization of proteins Examples of such causes of protein deficiency, occurring in patients to

whom adequate diets are economically available, would be obstructions or functional derangements of the gastrointestinal tract, and the metabolic alterations produced by infections, liver damage, or hyperthyroidism

Intravenous feeding with proteins is a directly practicable procedure if the protein used is a plasma protein preferably albumin, from the same species as the recipient Dogs can be kept in nitrogen balance by the intravenous injection of adequate amounts of dog plasma as the sole source of nitrogen Human plasma albumin in amounts of 37 5 grams daily by vein, has maintained nitrogen balance in human subjects for as long as 16 days (7), with no other nitrogenous food Larger amounts were necessary when plasma albumin was fed These results imply that amino acids stored as plasma protein are nutritionally available It is not yet clear whether or not complete hydrolysis of plasma protein precedes such nutritional utilization High cost makes intravenous alimentation with human albumin undesirable for general use Protein hydrolysates, which contain a mixture of amino acids are less effective weight for weight than plasma albumin in maintaining nitrogen balance but are definitely more economical

Hypoproteinosis of childhood Present-day American parents have been well indoctrinated with the necessity for supplying vitamins in the diets of their children and for supplying minerals, particularly calcium Lynch and Snively (19) report that many children seen in office practice have been fed chiefly on milk and carbohydrate food, to the neglect of meat and eggs Milk contains protein but in relatively dilute solution so that more than a quart a day is required for adequate protein intake in older children

Such underproteinized children tend to lack appetite to fail in normal growth, and to be subject to frequent gastrointestinal and other ailments Exclusion or strict limitation of milk, with insistence upon more concentrated forms of protein at every meal, has been a successful therapeutic approach

Effects of Prolonged Fasting

Terence MacSwiney the mayor of Cork, fasted 74 days in protest during his incarceration and died of starvation This is the longest period of complete fasting of which we have accurate knowledge as to the exact duration

Sunderman (31) has reported studies on a subject who had fasted 45 days The loss in weight during the fast was 40 5 pounds starting at 137 5 and ending at 97 No chemical studies were made before or during the fast which was undertaken for religious reasons Biochemical data at the close of the fast were compared with similar measurements made 43 days after customary food intake had been resumed Some of the results are shown in table 34 Note that at the end of the fast Cl^- had been lost from the blood

TABLE 31

Summary of observations on a man who had fasted 45 days

Date	Date in relation to day of fast	Body weight	Chloride	CO2	Inorganic phosphorus	Total base	Calcium	Magnesium	Total protein	Albumin	Globulin	Total cholesterol	Ester cholesterol	Nonprotein nitrogen	Uric acid	Creatinine	Sugar (serum)	Gonadotrophin	17 ketosteroid
		lb	mEq	vol %	mgm/100 ml	mEq/l	mgm/100 ml	mEq/l	gm/100 ml	gm/100 ml	gm/100 ml	mgm/100 ml	mgm/100 ml	mgm/100 ml	mgm/100 ml	mgm/100 ml	mgm/100 ml	mau/24 hrs	mgm/24 hr
4 1o	41st day of fast	101																	
4 1?	4?th day of fast		1 0	100	3 1	141	12												
4 21	5th day after fast	111	10? 8	63		146	10 7	1 6	5 4	? 8	2 6	1?5	1?4	?	? 0	1 1	? 3	×	
												—	—	14	1 1	1 0	—		
6 1	47th day after fast	131	98 7	61	—	148	9 8	1 9	6 7	4 0	2 ?	190	110	12	4 1	1 0	83	7 2	4 7
Normal range of values			91-104	5.–60	3 4	143 145	[illegible]	[illegible]	6-?	3 3	1 3 2	[illegible]	[illegible]	[illegible]	3 4	1 0-1	?–50	110 4	16 8 15

plasma and replaced by HCO_3^-, and that the blood cholesterol was almost entirely in the ester form

General Metabolic Phenomena of Starvation

Half of the world is chronically underfed This half of the world's population subsists on diets in which cereals or potatoes make up 80 to 100 per cent Such a diet contains an inadequate quantity of proteins, and the proteins there contained are of relatively poor nutritive quality In this large population group, there is found both *subnutrition*, which is the lack of an adequate quantity of food, and *malnutrition*, which involves deficiency of food factors

During starvation, the body, not receiving enough calories for maintenance of body structure and normal activity, draws upon its stores of fats and carbohydrates Survival under conditions of complete starvation depends, to a major degree, upon the amount of adipose tissue available Glycogen stores are usually exhausted within 24 hours As first the carbohydrates and then the fats are depleted, the body's proteins are progressively drawn upon and wasting of tissue occurs In early stages of starvation, decreases in the protein content of spleen, liver, and genitalia can be observed Skeletal muscle is drawn upon at later stages, while the heart, adrenals, and central nervous system show the least protein loss of all tissues Amino acids derived from less significant tissues are apparently used for the maintenance of these essential structures If starvation proceeds to the stage of loss of muscle proteins, creatine appears in increased amount in the urine

The basal metabolic rate in states of prolonged severe starvation decreases below the value predicted on the basis of age, weight, height, and similar criteria Such depressions of basal metabolic rate may fall as low as minus 30 per cent and still be reversible by adequate food intake, particularly of protein foods

Ketosis can be brought about by periods of fasting as short as 24 *hours*, and is certain to appear within the first few days of acute starvation Such ketosis is mild compared to that of severe diabetes, since the mechanisms for the oxidative utilization of foodstuffs are not impaired or overloaded, as they are in the diabetic After the exhaustion of stored glycogen, the body proteins may in part be converted into glycogen The ketone acids are excreted partly as ammonium salts and partly paired with alkali metal cations Sodium loss is most noticeable early in the ketosis of starvation Later there is potassium loss paralleling the atrophy of muscles and other tissues

As starvation progresses, other nitrogenous components of the body decrease along with the tissue proteins The blood plasma, for instance

which normally shows a total protein concentration consisting of about 4.7 per cent albumin and 2.5 per cent globulin (A/G ratio of about 1.9), shows values decreased to 2 to 3 per cent albumin and a globulin of around 2.5, yielding an A/G ratio of about 1.0 or less. It will be noted that the albumin decreases more than does the globulin, possibly reflecting a greater significance to the organism of the globulin fraction. A frequent and almost invariable consequence of severe protein deficiency is edema. This is often so pronounced that the victims, instead of being thin as expected, have distended abdomens. The development of such edema of starvation is in part the result of the lowered colloid osmotic pressure of the plasma, which in turn results from the decrease in plasma albumin. The non-protein nitrogenous constituents of the blood are not generaly affected, but may show moderate increase in prolonged starvation. This may, in part, be attributed to diminished renal function as a result of protein loss from the kidneys. The amino acid nitrogen of the blood remains surprisingly constant. Creatinine of blood or urine shows no consistent change, although creatinuria may occur as has already been mentioned.

The total nitrogen of the urine, of course, is much decreased since the source of the nitrogenous components is from protein metabolism. Urinary urea decreases along with the total excreted nitrogen, and in starvation is proportionally decreased even more, falling from a normal 90 per cent of the total to about 70 per cent. The ammonia in the urine is increased, consistent with the increase of excretion of ketone acids.

As the protein supplies of the body fall and with them the plasma protein level, it might be expected that the resistance of the body to disease would decrease, since antibodies (see Chapter 20) have been identified with the gamma globulins. Observations made during World War II on man did not bear out this contention. The body appears to continue to be able to produce antibodies even when it is prevented by inadequate protein supplies in the diet from making normal amounts of other proteins.

The exact mechanism of death by starvation has never been elucidated. It has been suggested that one of the main factors may be the inability to continue the production of proteins which form an essential part of the regular function of the body, such as nucleoproteins and protein hormones. In any case, it seems likely that the cause is directly or indirectly the failure of the supply of dietary proteins.

REFERENCES

1. Barron, E. S. G., and Flood, V. The oxidation of thiols by ionizing radiation. J. Gen. Physiol., 33: 229-241, 1950.
2. Borsook, H., et al. The formation of glycocyamine in man and its urinary excretion. J. Biol. Chem., 138: 405-410, 1941.

3 Borsook, H, and Dubnoff, J W The formation of glycocyamine in animal tissues J Biol Chem, 138: 389–403, 1941

4 Boyd W C *Fundamentals* [illegible]

5 Cc [illegible]

6 Du [illegible] biological synthesis of choline and creatine J Biol Chem, 140: 625–641, 1941

7 Eckhart, R D, *et al* Comparative studies on the nutritive value of orally and intravenously administered human serum albumin in man J Clin Investigation, 27: 119–134, 1948

8 Elliott, D F, and Neuberger, A Irreversibility of the deamination of threonine in the rabbit and rat Biochem J, 46: 207–210, 1950

9 Geiger, E Experiments with delayed supplementation of incomplete amino acid mixtures J Nutrition, 34: 97–111, 1947

10 Geiger, E The role of the time factor in feeding supplementary protein J Nutrition, 36: 813–819, 1948

11 Greenberg, D N, and Winnick, T The transformation in the rat of carbonyl labeled acetate, methyl labeled acetate, and labeled bicarbonate into amino acids Arch Biochem, 21: 166–176, 1949

12 Hill, C H, and Scott, M L Studies on the role of cysteine in the activation of folic acid conjugase J Biol Chem, 189: 651–658, 1951

13 Jolliffe, N, *et al* eds *Clinical Nutrition* New York, Paul B Hoeber, Inc, 1950

14 Keston, A S, and Wortis, S B The antagonistic action of choline and its triethyl analogue Proc Soc Exper Biol & Med, 61: 439–440, 1946

15 Keys, A, *et al* *The Biology of Human Starvation*, Vol I, pp 345–364 Minneapolis, Minn, University of Minnesota Press, 1950

16 Lerner, A B On the metabolism of phenylalanine and tyrosine J Biol Chem, 181: 281 294, 1949

17 Lignac, G O E A syndrome consisting of affections of the kidney, stunted growth, rickets, and disturbed cystine metabolism Am J M Sc 196: 542–547, 1938

18 Lundquist, F Splitting of phosphocreatine Nature, 159. 98–99, 1947

19 Lynch, H D, and Snively, W D, Jr Hypoproteinosis of childhood J A M A, 147. 115–119, 1951

20 Medes, G A new error of tyrosine metabolism Tyrosinosis The intermediary metabolism of tyrosine and phenylalanine Biochem J, 26 917–940 1932

21 Muntz, J A The inability of choline to transfer a methyl group directly to homocysteine for methionine formation J Biol Chem, 182: 489–499 1950

22 Ratner, S, and Pappas, A Biosynthesis of urea I Enzymatic mechanism of arginine synthesis from citrulline J Biol Chem, 179. 1183–1198, 1949

[illegible]

[illegible]

25 Rose, W C, *et al* The utilization of the nitrogen of ammonium salts, urea and certain other compounds in the synthesis of non essential amino acids *in vivo* J Biol Chem, 181. 307 316, 1949

26 Rosenthal R L *et al* Hematologic changes in rats protected by cysteine against total body x irradiation Am J Physiol, 166 15–19 1951

27 SCHOENHEIMER R *The Dynamic State of Body Constituents* Cambridge, Mass Harvard University Press 1942
28 SIEKEVITZ, P , AND GREENBERG D M The biological formation of formate from methyl compounds in liver slices J Biol Chem , 186 275–286 1950
29 STEKOL J A AND WEISS K *On de-ethylation of ethionine in the rat* J Biol Chem 185 577–583 1950
30 STETTEN M R Some aspects of the metabolism of hydroxyproline, studied with the aid of isotopic nitrogen J Biol Chem 181 31 37, 1949
31 SUNDERMAN F W Studies in serum electrolytes XIV Changes in blood and body fluids in prolonged fasting Am J Clin Path , 17, 169–180, 1947
32 TAGGART J V , AND KRAKAUR R B Studies on the cyclophorase system V The oxidation of proline and hydroxyproline J Biol Chem 177 641–653, 1949
33 TIERNEY N A , AND PETERS J P The mode of excretion of creatine and creatine metabolism in thyroid disease J Clin Investigation, 22 595–602, 1943
34 TORDA C AND WOLFF H G Glycocyamine elimination in patients with myasthenia gravis J Lab & Clin Med , 31, 1174–1178 1946
35 WEIL MALHERBE, H Significance of glutamic acid for the metabolism of nervous tissue Physiol Rev 30 549–568, 1950
36 WILLIAMS, J N , JR , AND ELVEHJEM, C A The effects of tryptophan deficiency upon enzyme activity in the rat J Biol Chem 183 539–544 1950
37 WITTENBERG, J , AND SHEMIN, D The location in protoporphyrin of the carbon atoms derived from the alpha carbon atom of glycine J Biol Chem , 185: *103–116, 1950*

CHAPTER 15

Electrolytes and Water: Edema and Shock

In addition to the carbon, hydrogen, nitrogen, and sulfur which, together with oxygen, make up the metabolic pathways discussed in the last few chapters, certain other elements are essential in the diet Absence or deficiency of these elements can check growth and cause specific losses of function These elements are *sodium*, *potassium*, *chlorine*, *calcium*, *magnesium*, *phosphorus*, *iron*, *copper*, *cobalt*, *iodine*, *fluorine*, *zinc*, and *manganese* These all can be utilized by the body if taken in the form of salts, hence can be expressed as ions To this list of metabolically significant ions should be added *ammonium*, *carbonate*, and *sulfate*, which are derived by metabolic action upon foodstuffs The *bromide* ion is present consistently in human plasma, but as far as we know it has no significance Minute and variable amounts of many other elements are present in the body without known function

Units of Concentration

Concentrations of these ions or of compounds involving them have been commonly stated in milligrams per 100 ml More convenient and useful units are the milliequivalent, the millimol, and the milliosmol The most useful of these is the *milliequivalent* An equivalent, meaning an equivalent weight in grams of an electrolyte or of an ion is the molecular weight of the compound, or the sum of the atomic weights of the atoms composing the ion, expressed in grams and divided by the electrovalence of the ion or by the number of electrons transferred in the formation of the electrovalent bond of the compound For example an equivalent of NaCl is 57 454 grams, one molecular weight, since only one electron is involved in its electrovalent bond Similarly an equivalent of Na^+, K^+, H^+, Cl^-, or Br^- is one atomic weight in grams, and the equivalent of NH_4^+ is the sum of the atomic weights in grams of one nitrogen and four hydrogens In the case of Na_2SO_4 the molecular weight in grams must be divided by two

to give the equivalent and the same division must be made in the case of bivalent ions like SO_4^{--} and Ca^{++} H_3PO_4 or $PO_4^{\equiv}$ must similarly have their weights divided by three to give the conventional equivalent We will see later that a compromise is made in expressing the equivalent concentration of phosphate in the blood plasma since this acid is never completely neutralized under physiological conditions The advantages of using this particular unit are (a) that one equivalent of any acid will exactly combine with one equivalent of any base to yield one equivalent of water and a total of one equivalent of salt or salts and (b) in calculation of total acidities or basicities of mixtures concentrations expressed in equivalents may be added together

The milliequivalent, being the thousandth part of an equivalent, is of a size better adapted for the expression of concentrations in blood and body fluids of electrolytes and their ions To convert the old time units (mgm per 100 ml) into milliequivalents (mEq) per liter, multiply by

$$\frac{10 \text{ number of electrons}}{\text{sum of atomic weights}}$$

It is now standard clinical practice to express concentrations of electrolytes and ions in mEq per liter and dosages of intravenous electrolytes in milliequivalents

The *mol* is defined as the molecular weight in grams, the *millimol* as the thousandth part of a mol These units are frequently used in expressing amounts and concentrations of non-electrolytes, particularly in physicochemical measurements

The *osmol* is the mol divided by the number of ions formed in the dissociation of the electrolyte in question, and the milliosmol is its thousandth part For non-electrolytes the osmol is equal to the mol The osmolar or milliosmolar concentration of a solution of known composition has a fixed and calculable value this value does not usually correspond exactly with the experimentally measured osmotic pressure The osmotic pressure developed experimentally depends not only upon the total osmolar concentration but also upon the degree of semipermeability of the experimental membrane to the solutes involved

The concept of *ionic strength* was defined in Chapter 1 It should perhaps be definitely stated that ionic strength is not identical with total ionic concentration expressed in any of the above units

Body Fluid Compartments

The total water of the body constitutes about seventy per cent of the weight of the fat free tissue or lean body mass of a normal adult Adipose tissue is low in water content so that a very obese subject might have as

low a water content as 35 per cent of body weight Total water can be estimated in small animals by desiccation at low temperatures In larger animals and man it is best measured by the intravenous injection of a known amount of a substance which is water soluble and to which all cell membranes are permeable The ideal test substances are the oxides of deuterium or tritium, but analytical procedures for these substances require elaborate instrumentation Of non isotopic test substances, antipyrine (74) has been most successfully used With either antipyrine or deuterium oxide, about two hours must be allowed for equilibration Then the concentration of the test substance is determined in the water of a sample of the subject s blood plasma

$$\text{Liters total body water} = \frac{\text{mgm test substance injected} - \text{mgm excreted}}{\text{mgm test substance per liter plasma water}}$$

The average lean adult will be found to contain about forty nine liters (70 per cent of 70 kgm) of total free body water If a substance to which cell membranes are not permeable, such as a bromide, is used as the test substance, a similar calculation will give the volume of *extracellular fluid* (6) In our same average adult this volume will be about fourteen liters Subtracting this from the total free body water we get 35 liters as the approximate volume of the *intracellular fluid* Again repeating the experiment and using as a test substance an organic dye which does not escape quickly from the blood vascular system, we can estimate the *blood plasma volume* In our same average lean subject this will be about three liters Subtracting this from the volume of extracellular fluid, we obtain about eleven liters, the volume of *interstitial fluid*—fluid which lies around the tissue cells but outside their walls and also outside the blood vessels To summarize we find in the average (70 kgm) lean adult

Intracellular fluid		35 liters
Extracellular fluid		
Plasma	3 liters	
Interstitial fluid	11 liters	
Total extracellular fluid		14 liters
Total free body water		49 liters

Normal Water Balance

Water is freely diffusible throughout the body and moves from one compartment to another as impelled by hydrostatic and osmotic forces The intracellular compartment (excluding blood cells and endothelial cells) can exchange only with interstitial fluid which in turn can exchange with plasma Water enters or leaves the body directly only to or from the plasma compartment Water is produced, however, within cells by the oxidation

of the hydrogen of metabolites: this is called metabolic water, or water of oxidation. Combining this with the interchanges of water between plasma and the outside world, a balance-sheet of water turnover can be set up. The approximate figures for our average 70 kgm. man would be:

Intake per 24 hours	liters	Output per 24 hours	liters
Fluids	1.2	Lungs	0.5
Water in food	1.0	Skin	0.5
Metabolic water	0.3	Urine	1.4
		Feces	0.1
Total	2.5		2.5

Although the figures given represent a purely hypothetical average subject, and even though the individual items are obviously subject to wide variation, certain significant facts are illustrated: 1) The normal person is in a state of water equilibrium, with neither a positive balance (water gain) nor a negative balance (water loss) over the 24 hours. 2) The state of water balance can not be accurately established by the crude comparison of fluids taken in and urine excreted.

The maintenance of water equilibrium in an infant is a more active physiological process than in the adult. Although a baby has a higher percentage of water in his body (80 per cent in the newborn as compared with 70 per cent in the adult), his body is so much smaller that ability to store water is sharply limited. The infant exchanges daily a much greater proportion of body water: a baby six months old and weighing about seven kgm. will have no more than 5.5 liters of total body water. His extracellular fluid will be about 1.4 liters and his daily exchange of water through intake and output about 0.7 liters. The infant at this age normally exchanges about half of his extracellular fluid during the 24 hours, the adult only about 18 per cent. Failure to ingest or retain fluids by the infant, or loss of body fluids by the infant, leads more quickly to a state of dehydration. Even moderate dehydration in a young child may cause fever; drastic restriction or loss of fluids impairs all physiological functions, particularly that of the kidneys. Pediatric experience has settled upon a total fluid intake of 2.5 fluid ounces per pound of body weight (166 ml. per kgm.) as an adequate intake for normal babies in temperate climates, increasing to 3 fluid ounces per pound (200 ml. per kgm.) in warm weather. Fortunately, the normal body protects itself well by renal function against water excess, so that even young infants can be allowed to take all the water they will. Feeding formulas are usually constructed on the 2.5 to 3 fluid ounce per pound basis, with a top limit at 35 fluid ounces (about a liter). A well baby who has reached this maximum can take additional water according to his needs.

In addition to the equilibrium of body water as determined by intake and

output, there is an osmotic equilibrium which concerns the several compartments Water can move freely from any compartment to one adjacent hence the compartments tend to have the same total osmotic pressure which has the rather astonishing value of 7 9 atmospheres or 6000 mm of mercury This enormous force is never available since many solutes also pass freely through compartmental partitions One substance which does not pass freely is protein The intracellular fluid is high in protein content and the cellular boundary is impermeable under physiological conditions to protein The outlying interstitial fluid is low in protein This difference *in concentration of a non diffusible* substance conditions an osmotic flow of water and diffusible solutes from interstitial fluid to the interior of the cell which is checked by the limit of the capacity of the cell A distensive force is thereby applied to the cell which is opposed by the compressive force of the stretched elastic cell boundary and the surrounding elastic *connective tissue In addition to water brought in by osmotic force* metabolic water is also produced within cells by the oxidation of the hydrogen of metabolites By successive slight alterations of the forces making up this equilibrium an ebb and flow of cell water can be postulated bearing with it soluble nutrients and waste products Assuming purely static conditions water and other substances to which the cell wall is permeable enter and leave the cell by diffusion

Exchanges between blood plasma and interstitial fluid are less restrained The capillaries are not uniformly impermeable to protein depending upon anatomical location the capillary wall varies from an almost complete barrier to proteins (as in the ciliary body of the eye) to a highly permeable meshwork (as in the hepatic sinusoids) The protein concentration of interstitial fluid is therefore variable in different organs but always less than that *of blood plasma This difference in colloid osmotic pressure* conditions an osmotic flow of water and diffusible solutes from interstitial fluid to plasma. This flow is augmented by tissue pressure and opposed by capillary blood pressure On account of frequent small variations in these pressures true equilibrium is probably never established in living tissues It seems legitimate to state that despite minor fluctuations the relative volumes of plasma and interstitial fluid remain within physiological limits as a result of a balance of these four pressures—colloid osmotic pressure of plasma and tissue pressure opposing capillary pressure and colloid osmotic pressure of the interstitial fluid

Since blood pressure is higher as arterial blood enters the capillary ejection of fluid occurs through the capillary wall Pressure *falls along the* capillary allowing a re entry of fluid as a result of osmotic force *It is important to consider this exchange of fluid as a rapid and dynamic turnover* rather than simply as a balance of pressures The rapidity of exchange

between plasma and extracellular fluid is indicated by the measurement of the diffusion of short life radioactive Na^{24} across the vascular wall (7). The results in human subjects indicated that 32 per cent of the total plasma Na^+ leaves the capillary blood every minute.

Dehydration and Thirst

When water intake is stopped, water output is diminished. The urinary volume will fall to about 500 ml. per 24 hours, but the output through skin and lungs will not be significantly altered. These unescapable water losses add up to about 1500 ml. per day. Since the channels of water loss lead directly from the plasma, this compartment will be the first to lose water; this water is replaced promptly from the interstitial fluid. Since water moves freely among the compartments, as soon as there is a significant increase in concentration of extracellular solutes, water will move from the intracellular fluid to the extracellular fluid as a result of the difference in concentration. Hence in water deprivation there will be loss of water from all compartments, *osmotic pressures will remain equal in all compartments*, but since the intracellular compartment contains the most water, it will lose the most.

An additional loss is borne by the intracellular compartment since potassium escapes from the dehydrating cells to the extracellular fluid and is eventually excreted in the urine. This has been demonstrated in dogs (23); to what extent this represents a loss in osmotic concentration within the cell and therefore contributes to loss of cellular water has not been fully determined either in dogs or men. A part, at least, of the lost cellular potassium is replaced by sodium, which normally is not present in significant amount in the cellular fluid. The chief intracellular cations are K^+ and Mg^{++}. Na^+ is the preponderant cation in the extracellular compartments and Cl^- is the chief anion.

Thirst is a sensation resulting from loss of cellular water (18). It is the chief symptom for the first two days of water deprivation in an otherwise healthy person, and increases in intensity with continuing water lack. By the third day dehydration of muscular and neural cells has reached a degree where such cells show functional changes, manifested in the patient by weakness and confusion. Disability increases with progressive cellular dehydration. *Death occurs when 15 per cent of the body weight has been lost*, usually within ten days of complete water deprivation.

Dehydration is a constant threat to the patient with *diabetes insipidus*. Reabsorption of water in the renal tubules fails in this disease on account of deficiency of the postpituitary antidiuretic substance. Verney (83) has shown in dogs that liberation of the antidiuretic substance from the neurohypophysis follows the stimulation of osmoreceptors in the central nervous

system by intracarotid injections of hypertonic solutions of NaCl This water conserving mechanism normally goes into action whenever the salt *concentration is increased* In *diabetes insipidus* this mechanism fails and water is maximally excreted regardless of the concentration of the blood plasma The daily urine volume exceeds 6 liters the specific gravity is 1 006 or less The patient becomes rapidly dehydrated, suffers intense thirst, and gains relief by drinking very large volumes of water Diabetes insipidus is evidence of damage by injury or disease to a functional unit which includes the posterior lobe of the pituitary, the paraventricular and supraoptic nuclei in the hypothalamus and the supraopticohypophyseal tract (20) The disease may result from damage to the osmoreceptors the postpituitary itself, or the interconnecting tract

Salt intoxication is comparable to water depletion Excretion of salt by the human kidneys requires a liter of water for each 300 mEq of sodium chloride excreted Drinking salt solutions of greater concentration than *this excretory limit results in loss of the water obligated in the excretion of* the excess salt The desperate expedient of augmenting scanty supply of drinking water with urine is therefore of no physiological advantage whatever The use of sea water for drinking in similar desperate situations is not only useless but actually harmful since 1) sea water is more concentrated than the physiological excretion limit so that extra water is removed from the body for its excretion, and 2) its use is likely to produce vomiting and diarrhea with extra water losses The drinking of alcoholic liquors under circumstances of water deficiency is also harmful, since alcohol produces water loss by inhibition of the pituitary antidiuretic mechanism (77) The symptoms of salt ingestion in excess of water intake are identical with those of water depletion and arise by the same mechanisms from loss of cellular fluid

Excessive sweating involves some loss of salt but a greater loss of water since sweat is a hypotonic solution with respect to plasma Sweating without compensatory water intake produces a situation comparable to water deprivation, and will accelerate the progress of the syndrome of water deprivation More commonly, *losses by sweating are made up* by increasing the water intake In this situation salt (NaCl) deficiency can develop A similar cause of salt deficiency is loss of *gastrointestinal fluids* when replacement is made by water without salt The salt concentration of the fluids lost by diarrhea, vomiting or surgical drainage is comparable to that of extracellular fluid *Deficiency of the adrenal* cortical hormones (Addison s disease) is characterized by excessive excretion of Na^+, in ketonuria from diabetes or starvation there is similar depletion since the ketone acids are excreted partly as sodium salts, and the loss may be *augmented by* vomiting polyuria from other causes, such as chronic nephritis may in

volve salt loss The purposeful restriction of sodium intake to very low levels and the use of potent diuretics to increase the salt excretion are valuable therapeutic procedures in the treatment of congestive heart failure Soloff and Zatuchni (75) have emphatically pointed out that this may lead to disastrous results in a small number of patients These patients are discovered to be in a *state* of drastic salt depletion with accompanying dehydration

Patients with *salt depletion* from any of the causes mentioned show a consistent grouping of symptoms In all instances, there is diminished volume of extracellular fluid both in the plasma and interstitial compartments There is no warning sensation comparable to thirst Urine volume is not consistently diminished as in water deprivation the Na^+ and Cl^- output in the urine is greatly decreased Early symptoms are weakness and faintness passing into stupor appetite is lost early while vomiting complicates the more advanced stages and adds to the salt loss If this vicious cycle is not checked, the patient goes into an apathetic confused state with decreased blood pressure and increased pulse rate Death results from the diminished volume and increased viscosity of the blood

Water intoxication (heat cramps or miner's cramps) occurs when much water is taken by a person with salt depletion or without compensatory salt intake The usual story is one of rapidly drinking large volumes of water after severe or protracted sweating It has been reported in hospital patients who have been given dilute (isotonic) glucose solution intravenously faster than their kidneys could handle the water The cells acquire excess water, while extracellular fluid volume is diminished (55) Prevention and treatment consist of replacing the deficient salt

Edema

Edema is the retention of both water and electrolytes—an increase in the volume of interstitial fluid In amount it may involve as much as 100 pounds of water and a pound of sodium chloride (62) It may be local or general, it may permeate connective tissue or accumulate in cavities Numerous physical and chemical mechanisms are known to have a part in its formation These mechanisms may act singly or in any combination

Increased venous and capillary blood pressure may result from cardiac insufficiency or venous obstruction Excessive fluid is filtered through the capillary wall Edema of cardiac origin is characterized by its mobility—it responds to gravity and sinks to the lower parts of the body, shifting slowly after changes of position It may also gather in the pleural and peritoneal cavities The localization of the edema of venous obstruction is determined by the site of the obstruction, and is less influenced by gravity *Ascites*, accumulation of fluid in the peritoneal cavity in cirrhosis of the liver is

in part the result of *constriction of the portal circulatory bed by scar* tissue which has replaced normal liver structure

Decreased plasma protein diminishes the colloid osmotic pressure of the plasma and permits *greater net outflow to the interstitial fluid* This mechanism is also operative in cirrhosis of the liver On account of its greater concentration in plasma and lower molecular weight, albumin has more osmotic effect than globulin *In cirrhosis and other diseases involving* destruction of liver cells, plasma albumin decreases faster than globulin Typically a hypoproteinemia develops, with increased proportion of globulin to albumin—in clinical jargon, a decreased or reversed A/G ratio This mechanism is probably responsible for ascites in early stages of cirrhosis, portal obstruction adds to the difficulty later

The edema of *malnutrition* is also the result of failure to produce plasma albumin, in this instance the lack is of protein food The plasma volume is diminished, which gives to the blood analyst a falsely optimistic view of the nutritional state by partly compensating for the greatly depressed levels of albumin, hemoglobin, and red cells

The *nephrotic syndrome* is characterized by low levels of plasma protein and the excretion of large amounts of albumin in the urine The syndrome may exist by itself, or as a stage in the development of several varieties of renal disease Nephrotic edema is widespread through the body, as indicated by measurements of extracellular fluid volume The rate of protein loss in the urine is usually adequate to explain the hypoproteinemia and the resulting edema, but this is not always the case and there are a number of unresolved questions about this disease (11)

Decreased elimination of water and electrolytes occurs in acute nephritis as a result of decreased glomerular filtration and in cardiac insufficiency as a result of decreased renal blood supply The ions involved are those of extracellular fluid particularly sodium

Increased permeability to protein of the capillary wall effectively decreases the colloid osmotic pressure of the plasma even in the absence of hypoproteinemia *This is probably the mechanism of the formation of sharply* localized areas of edema, for example, the ordinary mosquito bite or the sudden angioneurotic edema which is a common manifestation of clinical allergies *It may also take part in the formation of the edema of nephritis* in this disease there are visible signs of damage to blood vessels

Lymphatic obstruction causes localized edema, the fluid is usually high in

moves by force of gravity or of increased tissue pressure

structed area is promptly absorbed

It is plain that the multiplex disturbances which produce edema can not be corrected by any single therapeutic measure In a particular patient, several of the mechanisms may be working together A malnourished patient for example, may be edematous both from hypoproteinemia and from cardiac insufficiency of thiamine deficiency Aside from the correction of dietary deficiencies, effective therapeutic measures include 1) improvement of cardiac efficiency by adequate nutrition or by cardiac drugs such as digitalis 2) decrease of demand on the heart muscle by restricting activity 3) restoration of plasma protein by injection of human plasma, or better of plasma albumin concentrate (even in nephrotic edema where albumin is being wasted this may decrease gastrointestinal edema and improve the appetite for and digestion of protein foods), 4 restriction of salt intake 5) the use of diuretic drugs to increase urinary output of water and salts 6) chemotherapy and other effective measures against infection 7) surgical removal or by passing of venous or lymphatic obstruction, and 8) surgical removal of collections of edema fluid

Shock

Shock is a state in which all physiological functions are depressed and disorganized as a result of failure of adequate arterial blood supply from the heart Shock can arise from many causes most of which are physical rather than chemical in their nature The changes produced by shock are both physical and chemical Any form of damage to the body which can result in a decrease of the general arterial blood supply can cause shock Blalock has classified shock according to the physiological system responsible for the primary disturbance (a) hematogenic shock results from loss of circulating blood and the operative mechanism is the reduction of blood volume (b) neurogenic shock results from physical damage to the nervous system or from the action of drugs upon the nervous system as in spinal anesthesia with the reduction of blood pressure the primary operative mechanism (c) vasogenic shock follows vasodilatation mediated otherwise than through neural mechanisms with lowering of blood pressure again the effective mechanism as in histamine shock or anaphylactic shock (d) cardiogenic shock where again lowered blood pressure is the effective mechanism but the cause is failure of the pumping action of the heart Combinations of these mechanisms may occur Vasogenic shock may complicate any other type of shock by the release from liver and muscle under hypoxia of a vasodepressor material (VDM) which has been identified as ferritin Whether the blood pressure fails or the blood volume the series of events which follows is similar The failure of normal arterial blood supply leads to stagnant hypoxia There is an immediate depression of all

physiological functions Most notably, consciousness is lost and body temperature becomes subnormal With continued *hypoxia and metabolic depression*, if the patient survives there is a predominance of hydrolytic and anaerobic chemical processes and a depression of the anabolic synthetic and oxidative mechanisms Blood sugar rises at first from the breakdown of glycogen, the rate of glycogenolysis being augmented by the liberation of adrenalin *Anaerobic glycolysis continues, but since the oxidative functions are depressed*, there is accumulation of lactate and pyruvate The highly energized adenosine polyphosphates diminish in all tissues particularly the liver and kidney Renal function is depressed as indicated by increased levels of non protein nitrogen in the circulating blood while the *accumulation of acid metabolites tends to depress the pH of blood and body fluids* Disintegration of tissue cells is evidenced by liberation of potassium a typically intracellular ion into the extracellular fluids and the blood plasma and by an increased content of the blood in polypeptides Hepatic hypoxia depresses the deamination of amino acids During this *stage of tissue breakdown the body temperature tends to rise from its* initial depressed level and usually to go above the normal There is no evidence of extensive loss of cellular water in shock Increase of plasma volume at the expense of interstitial fluid is a physiological response in compensatory opposition to the mechanisms which originally induced the shock state *Hemodilution is therefore considered a favorable sign in shock* patients It should be emphasized that even where there is no external bleeding, traumatic shock results in a primary loss of blood volume In burns, plasma is lost by exudation through the burned area in contused or crushed regions of the body, fluid is constantly escaping from the capillaries *and accumulating in the injured region Prediction of the water balance* subsequent to the appearance of shock is unreliable Extra fluid losses may occur through sweating increased respiratory rate, and occasionally by vomiting On the other hand the depression of renal function usually keeps urinary outputs at a low value

The cardinal points in the treatment of a patient in shock are a prompt decision implicating the physiological disturbances contributory to the shock state and the quickest possible restoration of adequate arterial blood supply The longer a patient remains in a state of shock *the greater* is the probability that the physiological and biochemical changes may *become irreversible It is* of course, imperative to check or minimize hemorrhage as promptly as possible The use of the head-down shock or Trendelenburg position usually brings about an elevation of blood pressure which is desirable Restoration of *blood volume should be accomplished as* promptly as possible, preferably with whole blood second best with blood

derivatives such as human blood plasma, human plasma albumin, human red cell suspension, or human ascitic fluid. The use of simple glucose or saline solutions, if blood or blood derivatives are not available, may restore consciousness and reverse the direction of the unfavorable physiological changes. Since these simple solutions do not contribute to the colloid osmotic pressure of the plasma, the benefits derived from their use are likely to be transitory. Numerous "blood substitutes" with a colloidal component such as gum acacia or a protein of non human origin have been used experimentally, none of them has proved fully acceptable. Restoration of normal blood pressure and normal blood volume is the single major therapeutic objective in the immediate treatment of shock. Much else can be and should be done for the benefit and comfort of the shock patient, for such measures and for detailed discussion of the principles and techniques of blood volume restoration, the student should consult textbooks of medicine and surgery and also specialized monographs on shock (17, 80).

Analysis of Compartmental Fluids

It is a simple matter to obtain a sample of blood plasma. It is next to impossible to secure samples of normal human interstitial fluid or of the intracellular fluid of tissues; analysis of these fluids has been by indirection. (a) Direct analysis of such cells as those of blood, pus, or sperm, which can be isolated from their surrounding fluids, indicates a fundamental difference in mineral composition from that of blood plasma. Na^+ and Cl^- are the chief ions of blood plasma; these ions are absent or in low concentration in the cells—in their place are found K^+ and HPO_4^{--}. This difference between cell and surrounding fluid, although subject to great quantitative variations, is consistent in all forms of life and in the lifeless earth. "Potassium is of the soil and not the sea, it is of the cell and not the sap" (28). (b) By making the assumptions that Cl^- is limited to extracellular fluid and that the concentration of Cl^- is the same in plasma and in extracellular fluid, then

$$\frac{\text{Total mEq } Cl^- \text{ in tissue sample}}{\text{mEq } Cl^- \text{ per liter plasma}} =$$

Liters extracellular fluid in tissue sample

(In practice, since we know that both assumptions are inexact, corrections are applied to both numerator and denominator of the above fraction in accordance with our knowledge. In muscle we know, for example, that there appears to be 3 mEq of Cl^- per liter of intracellular water.) Knowing the volume of extracellular fluid in the sample, and still assuming uniform distribution of ions throughout the extracellular fluid, we can work out

physiological functions Most notably consciousness is lost and body temperature becomes subnormal With continued hypoxia and metabolic depression, if the patient survives, there is a predominance of hydrolytic and anaerobic chemical processes and a depression of the anabolic synthetic and oxidative mechanisms Blood sugar rises at first from the breakdown of glycogen the rate of glycogenolysis being augmented by the liberation of adrenalin Anaerobic glycolysis continues but since the oxidative functions are depressed there is accumulation of lactate and pyruvate The highly energized adenosine polyphosphates diminish in all tissues particularly the liver and kidney Renal function is depressed as indicated by increased levels of non protein nitrogen in the circulating blood while the accumulation of acid metabolites tends to depress the pH of blood and body fluids Disintegration of tissue cells is evidenced by liberation of potassium a typically intracellular ion into the extracellular fluids and the blood plasma and by an increased content of the blood in polypeptides Hepatic hypoxia depresses the deamination of amino acids During this stage of tissue breakdown the body temperature tends to rise from its initial depressed level and usually to go above the normal There is no evidence of extensive loss of cellular water in shock Increase of plasma volume at the expense of interstitial fluid is a physiological response in compensatory opposition to the mechanisms which originally induced the shock state Hemodilution is therefore considered a favorable sign in shock patients It should be emphasized that even where there is no external bleeding, traumatic shock results in a primary loss of blood volume In burns, plasma is lost by exudation through the burned area in contused or crushed regions of the body fluid is constantly escaping from the capillaries and accumulating in the injured region Prediction of the water balance subsequent to the appearance of shock is unreliable Extra fluid losses may occur through sweating increased respiratory rate and occasionally by vomiting On the other hand the depression of renal function usually keeps urinary outputs at a low value

The cardinal points in the treatment of a patient in shock are a prompt decision implicating the physiological disturbances contributory to the shock state and the quickest possible restoration of adequate arterial blood supply The longer a patient remains in a state of shock the greater is the probability that the physiological and biochemical changes may become irreversible It is of course, imperative to check or minimize hemorrhage as promptly as possible The use of the head-down shock or Trendelenburg position usually brings about an elevation of blood pressure which is desirable Restoration of blood volume should be accomplished as promptly as possible preferably with whole blood second best with blood

derivatives such as human blood plasma, human plasma albumin, human red cell suspension, or human ascitic fluid The use of simple glucose or saline solutions, if blood or blood derivatives are not available, may restore consciousness and reverse the direction of the unfavorable physiological changes Since these simple solutions do not contribute to the colloid osmotic pressure of the plasma, the benefits derived from their use are likely to be transitory Numerous "blood substitutes" with a colloidal component such as gum acacia or a protein of non human origin have been used experimentally, none of them has proved fully acceptable Restoration of normal blood pressure and normal blood volume is the single major therapeutic objective in the immediate treatment of shock Much else can be and should be done for the benefit and comfort of the shock patient, for such measures and for detailed discussion of the principles and techniques of blood volume restoration, the student should consult textbooks of medicine and surgery and also specialized monographs on shock (17, 86)

Analysis of Compartmental Fluids

It is a simple matter to obtain a sample of blood plasma It is next to impossible to secure samples of normal human interstitial fluid or of the intracellular fluid of tissues analysis of these fluids has been by indirection (a) Direct analysis of such cells as those of blood, pus, or sperm, which can be isolated from their surrounding fluids, indicates a fundamental difference in mineral composition from that of blood plasma Na^+ and Cl^- are the chief ions of blood plasma these ions are absent or in low concentration in the cells—in their place are found K^+ and HPO_4^{--} This difference between cell and surrounding fluid, although subject to great quantitative variations, is consistent in all forms of life and in the lifeless earth "Potassium is of the soil and not the sea, it is of the cell and not the sap" (28) (b) By making the assumptions that Cl is limited to extracellular fluid and that the concentration of Cl^- is the same in plasma and in extracellular fluid, then

$$\frac{\text{Total mEq } Cl^- \text{ in tissue sample}}{\text{mEq } Cl^- \text{ per liter plasma}} = \text{Liters extracellular fluid in tissue sample}$$

(In practice, since we know that both assumptions are inexact, corrections are applied to both numerator and denominator of the above fraction in accordance with our knowledge In muscle we know, for example, that there appears to be 3 mEq of Cl^- per liter of intracellular water) Knowing the volume of extracellular fluid in the sample, and still assuming uniform distribution of ions throughout the extracellular fluid, we can work out

the partition of any ion, such as Mg^{++}, as follows

mEq extracellular Mg^{++} in sample =
(liters extracellular fluid in sample) × (mEq Mg^{++} per liter plasma)

and

mEq intracellular Mg^{++} in sample =
(total mEq Mg^{++} in sample) − (mEq extracellular Mg^{++} in sample)

(c) The direct analysis of ultrafiltrates of plasma or of normal or pathological fluids which are derived from the blood plasma by filtration through

TABLE 35

Approximate concentrations of osmotically significant ions in human body fluids milliequivalents per liter of water

	Na	K^+	Ca^{++}	Mg^{++}	PROTEIN	ORGANIC ACIDS	Cl	HCO_3	HPO_4	SO_4
Blood plasma	144	4.5	5.0	1.8	18	6	105	28	2.0	0.7
Cerebrospinal fluid	151	4.0	3.0	5.0			125	21		
Gastric juice	20	8					146			
Bile	142	8					109	38		
Pancreatic juice	141	5					40	111		
Jejunal juice	140	6					111	30		
Sweat	83	5	5	5			86			
Edema fluid	135	4.6								
Pleural fluid	137	4.3								
Intracellular fluids										
Skeletal muscle	7	155			74		3	60		
Cardiac muscle	15	142			75		7	67		
Liver	5	145			89		12	98		
Brain	27	184			46		3	102		

capillary walls (cerebrospinal fluid, aqueous humor, glomerular fluid) or which are accumulations of interstitial fluid (ascitic fluid, lymph, blister fluid) yields varying results, since each of these fluids has a different physiological history after it leaves the blood vascular system There is a certain consistency about their composition which reflects their common origin Average results of analyses by the above methods of specimens of human origin are shown in table 35, together with figures on the ions of digestive juices The data were compiled from a number of sources (16, 33, 44, 50, 60, 61, 71) The conventions used in calculating mEq are those indicated by Gamble (35) for HPO_4^{--} the two electrons are calculated as 1 8 since 20 per cent of the ion is in the form of $H_2PO_4^-$ at pH 7 4, the Van Slyke factor, 2 43 is used to convert per cent protein to mEq per liter, the factor for converting volumes per cent CO_2 to mEq HCO_3^- per liter

is 0.45. Conversion of units per liter serum to units per liter serum water is by use of the factor 1.07 for serum of normal specific gravity.

The small differences in concentrations of electrolytes in plasma-derived fluids as compared with each other and as compared with plasma can be explained in part by differences in protein content. While it is doubtful that a true Gibbs-Donnan equilibrium (see Chapter 1 and Appendix 1) is ever achieved in the body, the conditions for approaching such an equilibrium exist at the capillary boundary, where plasma of high protein content is separated from interstitial fluid of low protein content by a membrane of limited permeability to protein. The inequality of concentrations of the non-diffusible protein anion demands an unequal distribution of diffusible ions, assuming complete non-diffusibility of protein, the calculated distribution ratio

$$r_{sf} = \frac{\text{serum anion concentration}}{\text{fluid anion concentration}} = \frac{\text{fluid cation concentration}}{\text{serum cation concentration}}$$

is 0.96, which is approximated closely by Na^+, Cl^-, and HCO_3^-. The ratio for K^+ averages 0.92, indicating that it also can be considered freely diffusible. The theory predicts that the diffusible cations will be less concentrated and the diffusible anions more concentrated in the derived fluid than in the original plasma. With K^+, Na^+, HCO_3^-, and Cl^- this is the case in edema fluid, in accumulations of pleural and pericardial fluid, and in plasma ultrafiltrates.

Because of their clinical usefulness, the figures in table 35 for blood plasma should be permanently and exactly memorized and the others remembered as larger or smaller in comparison. No attempt has been made to maneuver the average figures in the table to make total anions equal total cations, in an actual fluid this must be so. The apparent ion deficit in gastric juice is made up by H^+, in pancreatic juice by OH^-; deficits in the neutral fluids may be blamed upon inadequacies of analytical and statistical methods.

SODIUM

Our daily Na^+ intake is approximately five grams or 220 mEq., and in a healthy person output matches intake. This daily turnover of Na^+, which depends almost entirely upon the Na^+ content of the diet, is subject to great variation as a result of individual tastes and habits. Restriction of Na^+ intake has been carried to the extreme of 150 mgm. per day in the rice diet (54). Under this regime the urinary Na^+ output falls to about 10 mgm. per day. The concentration of Na^+ in the blood serum does not significantly change but the blood plasma volume diminishes. With increased Na^+ intake the rough parallelism of output with intake is maintained up to

about three times the average figures stated The excretion of such large amounts of Na^+ requires an increase in water excretion above the normal which is approximately proportional to the excess Na^+ excreted Water taken with salt is however, excreted more slowly than water taken alone The experimental use of salt intakes of the order of 480 mEq per day caused a temporary gain of weight by water retention even though the urinary output increased to about 3 liters per 24 hours (7) The maximum concentration at which the human kidney can excrete sodium chloride seems to be about 300 mEq per liter Ingestion of sea water or of any saline solution above this critical concentration will require that water be removed from the body in the excretory process

Analysis in Blood Serum

The mean value for sodium in the serum of normal individuals has been determined in our laboratory as 145 mEq per liter with a standard deviation of 2 2 mEq per liter The method used (15) consisted of precipitation as uranyl zinc sodium acetate and color development with sulfosalicylic acid and sodium acetate This is but one of numerous modifications of a standard gravimetric method for sodium analysis Most clinical analyses for Na^+ were done by some modification of the uranyl zinc sodium acetate method until the introduction of the flame photometer This is an instrument specially designed to measure the energy of the atomic line spectra of characteristic wave lengths emitted by sodium and potassium when excited by high temperatures When properly standardized this instrument yields analytical results concordant with those obtained by the more tedious chemical examination and with a considerable saving of time In a series of 107 normal specimens, a mean value of 144 mEq of Na^+ per liter was obtained with a standard deviation of 3 6 mEq per liter (61) The Na^+ content of cerebrospinal fluid aqueous humor or other extracellular fluids available for analysis approximates 96 per cent of the plasma value as predicted by the Gibbs Donnan theory It has already been pointed out that the intracellular fluids are low and variable in Na^+ content

From the established Na^+ content of the extracellular fluid and from its estimated total volume about 14 liters we can easily calculate that the total Na^+ in the extracellular fluid of the body will be a little over two equivalents Actual analysis of the ash of the human body indicates the presence of nearly one equivalent more This neglecting the small intracellular fraction of Na^+ is located in the skeleton The mineral reinforcement of bone contains about one mol of Na^+ for 30 mols of Ca^{++} In summary the total body Na^+ which for our average man is about 60 grams or a little less than three equivalents is divided approximately one third in

the bones and two thirds in the extracellular fluids a small and variable amount of Na^+ is found within the cells

Clinical Significance of Sodium Analyses

The previous discussion has emphasized the constancy of the Na^+ content of blood plasma and extracellular fluid even under conditions of physiological stress Variation in either direction beyond the normal limit of Na^+ concentration is indicative of serious disturbance of either the osmotic balance or the acid base balance The value of Na^+ analyses in estimating disturbances of acid base balances will be considered in the next chapter As far as osmotic balance is concerned it will be recalled that shifts of water generally occur in such a way as to maintain the Na^+ concentration of the extracellular fluid and that the actual concentration of Na^+ will not change until the imbalance is excessive Hence the finding of a depressed level of plasma Na^+ is indicative of a severe degree of salt depletion or water excess Conversely, it is only in extreme states of water deficiency or salt excess that an elevated Na^+ concentration will be observed in the blood serum When such findings are noted an emergency exists and prompt correction is indicated For example if a person has been losing electrolytes by excessive diarrhea or vomiting to such a degree that the serum Na^+ is depressed it would be futile and dangerous to attempt to restore the volume of extracellular fluid with 5 per cent glucose solution From the point of view of osmotic balances this would be the equivalent of the injection of pure water Diuresis would occur with the loss of still more electrolyte The indication here is the restoration of fluid volume with [illegible]

tary salt restriction excessive use of diuretic drugs and adrenocortical deficiency In Addison s disease, as a result of a deficient supply of mineralocorticoids the concentration of Na^+ and also of Cl in the blood serum is distinctly below normal The plasma volume is greatly decreased in terminal cases as much as 45 per cent The concentration and total excretion of the sodium and chloride ions are greatly increased in the urine and also in the sweat The opposite situation to Addison s disease is hypercorticoadrenalism in which situation increased concentrations of Na^+ in the blood serum have sometimes been observed

POTASSIUM

The predominance of Na^+ among the cations of extracellular fluids yields to K^+ within the boundaries of the cells The observed fact that cells

accumulate K^+ in preference to Na^+ has not been adequately explained. Among the mechanisms involved (72) are the binding of K^+ to cellular lipids and proteins, and the active extrusion of Na^+ In human red cells K^+ accumulation is most effective at pH 7 4 and in glucose concentrations of 20 to 200 mgm per 100 ml Procedures which check cell metabolism e g heavy x irradiation or poisoning with fluoride, abolish the ability to accumulate K^+ beyond the small amount explained by Gibbs Donnan equilibrium Concentrations of K^+ vary in different tissues, rates of uptake measured by the use of radioactive K^{42}, also vary, uptake is more rapid in growing cells, with disintegration of cells K^+ is released, in general the concentration in milliequivalents of K^+ within cells is comparable to that of Na^+ *in the extracellular fluid (table 35) The approximate proportion to* protein in muscle is 3 mEq K^+ per gram of nitrogen The total potassium in the body of the average man is 4 75 equivalents or 185 grams the daily turnover averages about 80 mEq, ranging between 2 5 and 4 grams, more than four fifths of the daily excretion is in the urine, the remainder in the stools and sweat

It can be shown in experimental animals that there is a loss of K^+ from nerve during conduction The excitability of nerve endings in the frog increases with greater concentration of K^+ within the fiber as compared with the fluid outside The maintenance of such a difference in concentration is dependent upon continued cell metabolism The K^+ content in the brain of rats decreases in response to stress such as prolonged swimming The K^+ excretion of normal men increases in response to a wide variety of stresses, accompanied by an increase in exertion of corticoids and by other evidence of increased adrenocortical activity Such fluctuations in response to stress are minimal in schizophrenic patients (46)

The body contains more K^+ than Na^+ at all ages The difference becomes greater with growth Human milk contains 11 mEq of K^+ and 5 mEq of Na^+ per liter this ratio appears to be optimal or nearly so for the growth of the infant The ratio in meat is similar A mixed adult diet, including added salt, yields about 80 mEq of K^+ to 220 mEq of Na^+ Purely vegetarian diets have a greater proportion of K^+, their use requires heavier supplementation with Na^+ to maintain extracellular fluid volume The

extreme in this direction is diet already discussed by use of which

decrease in blood plasma v be induced

The blood plasma conta Eq of K^+ per liter with a standard

deviation of 0 45 mEq *(61)* ed by the flame photometer These

vali good agreeme rs previousl by tedious

and mical micro r values observed in

aqu and cerebr rough with Gibbs-

Do Blood mEq whole blood

kept in storage at low temperatures loses K^+ from cells to plasma, while hemolysis liberates large quantities It is essential for potassium analyses that serum be used which is unhemolyzed and which was promptly separated from the cells When blood is stored at 37°C, K^+ enters the cell from the plasma until glycolysis is complete, then reverses the direction of its movement Addition of glucose prolongs the retention of K^+ by red cells (14)

Potassium Deficiency

It is not possible to diagnose a state of K^+ deficiency with certainty on [illegible] ... somewhat was depressed when [illegible]

large quantities of gastrointestinal fluid, and who have been maintained chiefly by parenteral fluids with minimal K^+ intake, will retain much larger amounts This is taken to indicate K^+ deficit K^+ depletion occurs with restricted intakes since renal excretion of K^+ never falls to levels as low as those of Na^+ with minimal intakes The minimal excretion observed under complete K^+ deprivation was 6 mEq per day—compare this with the less than 0 5 mEq of Na^+ excreted on the rice diet

Water depletion has been shown to bring about loss of intracellular K^+ in dogs This is associated with the loss of intracellular water already discussed under water deficiency Renal excretion is a necessary mechanism for this loss of K^+ (23)

The concentration of K^+ in the extracellular fluid can be decreased by an increase in the volume of that compartment, a common example is the administration of fluids to a patient depleted of water and electrolyte If there is adequate cellular K^+, some will move out from the cells and restore normal extracellular concentration, in cases where there is deficit of cellular K^+, this adjustment may be inadequate (16)

It is difficult to assess the total results of K^+ deficiency in human patients, where the deficiency is usually a complication and not a primary disease It occurs most frequently in patients who have been parenterally nourished Since K^+ is present in all cells, animal or vegetable, and in milk, no diet intended to be eaten in the normal manner is likely to be deficient in this ion unless purposely made so out of purified materials Rats maintained on such a diet are apathetic, dwarfed, and diarrheic, the heart muscle degenerates and voluntary muscles are weak or paralyzed, the final stage is paralysis of the intestinal musculature Darrow (16) attributes the paralysis of hypokaliemic states to an effect upon the myoneural junction

Familial periodic paralysis is an inborn error of K^+ metabolism in which at unpredictable intervals the patient, usually during sleep, develops flaccid paralysis of the muscles of the trunk and extremities, lasting up to a few days Plasma K^+ concentrations during attacks fall to about half the normal value There is no unusual excretion of K^+, what appears to occur is a movement of K^+ from extracellular to intracellular fluid Five to 10 grams of KCl taken by mouth will terminate attacks within 2 hours, with restoration of plasma K^+ to normal Attacks may be prevented by the nightly use of a 5 gram dose of KCl Intravenous injection of one gram KCl in 50 ml water has promptly relieved dangerous paralysis of the muscles of respiration (69)

Potassium intoxication can occur when the concentration of K in the plasma is elevated above 7 mEq per liter (53) This may occur in Addison's disease, in severe renal inadequacy, and as a result of overdosage of potassium salts The chief effect is upon the function of the heart muscle and is best demonstrated in electrocardiograms The characteristic sequence of changes from the normal is 1) increased height and steepness of the T wave, 2) increased width of the QRS complex, 3) widening and later loss of the P wave, 4) gross intraventricular conduction defects and cardiac arrest with irregular waves of low voltage Useful therapeutic measures include the attempt to restore electrolyte [illegible] ce by intravenous injection of hypertonic (3 per cent) NaCl solutio[illegible] onic (20 per glucose solution containing one unit r[illegible] and more fundamentally the removal [illegible] fu iction Numerous mechanical devices (see page 676) have been used, and ma[illegible] is not irreversible

It is difficult to provoke K^+ intoxic[illegible] renal function is normal Where renal [illegible] to avoid the use of potassium compoun[illegible] high K^+ [illegible] ntent The manifestations o[illegible] result of [illegible] K^+ concentration [illegible] cellular flu[illegible] not to increased K^+ [illegible] experimental [illegible] one can increase [illegible] K^+ salts If wa[illegible] ble, the int[illegible] to normal A m[illegible] e in c[illegible] stages of experim[illegible] insuffici[illegible] also elevated (16)

RID

The 70 kgm adult con[illegible] significant portion of which

bination It is chiefly in the extracellular fluid the red cells of the blood are rich in chloride in comparison to tissue cells their Cl^- content is 77 to 91 mEq per liter The Cl^- of venous blood plasma is 99 to 105 mEq per liter with a mean of 102 mEq, values for arterial plasma are consistently 3 mEq higher—this is the chloride shift, a significant mechanism in CO_2 transport which will be explained in the next chapter The Cl^- content of whole blood depends upon the proportions of cells and plasma plasma or serum analyses for Cl^- may be clinically useful whole blood Cl^- is not

The daily turnover of Cl^- is even more variable than that of Na^+ The chief determinant is the NaCl of the diet which varies from negligible amounts in the rice diet up to 15 grams daily for a heavy salt eater, expressed as Cl^- this would be 9 grams or 330 mEq In the absence of sweating, normal excretion of Cl^- is almost entirely in the urine the daily fecal output is only about 3 mEq

The chloride of extracellular fluids (table 35) approximates the concentration predicted by the Gibbs Donnan equilibrium In the cerebrospinal fluid the Cl^- content is 119 to 128 mEq per liter which is higher than the predicted value With pathological increase of protein content as in tuberculous meningitis, the spinal fluid Cl^- decreases

Bromide

In the blood of dogs or men on an ordinary diet there is almost always a detectable amount of bromide the concentration is 0 1 mEq per liter or less This ion serves no known function in the body Its presence becomes medically significant only when the concentration is considerably increased When a bromide is taken in excessive doses or over a prolonged period of time for its sedative effect its concentration in the blood plasma may reach levels of from 10 to 20 mEq per liter At these levels impairment of the patient's mental functions, particularly memory and concentration, is likely to appear, often accompanied by a spotty purplish discoloration of the skin with elevation very similar to the papules of ordinary acne With even more enthusiastic use of the drug, still higher concentrations of (or culting bromide may be achieved, as an accompaniment of which the patient shows definite psychotic changes including disorientation, lethargy, and confusion There is an unpredictable effect upon reflexes usually shown by disturbances of gait and co-ordination Recovery from such bromide intoxication is usually spontaneous within a few weeks if the drug is no longer taken The distribution of bromide in the body is identical with that of chloride It will be recalled that bromide is commonly used to measure the volume of the extracellular fluid space Excretion of bromide and recovery from bromide intoxication can be accelerated by the administration of sodium or ammonium chloride (12)

Familial periodic paralysis is an inborn error of K^+ metabolism in which at unpredictable intervals the patient, usually during sleep, develops flaccid paralysis of the muscles of the trunk and extremities, lasting up to a few days Plasma K^+ concentrations during attacks fall to about half their normal value There is no unusual excretion of K^+, what appears to occur is a movement of K^+ from extracellular to intracellular fluid Five to 10 grams of KCl taken by mouth will terminate attacks within 2 hours, with restoration of plasma K^+ to normal Attacks may be prevented by the nightly use of a 5 gram dose of KCl Intravenous injection of one gram KCl in 50 ml water has promptly relieved dangerous paralysis of the muscles of respiration (C9)

Potassium intoxication can occur when the concentration of K^+ in the plasma is elevated above 7 mEq per liter (53) This may occur in Addison's disease, in severe renal inadequacy, and as a result of overdosage of potassium salts The chief effect is upon the function of the heart muscle and is best demonstrated in electrocardiograms The characteristic sequence of changes from the normal is 1) increased height and steepness of the T wave, 2) increased width of the QRS complex, 3) widening and later loss of the P wave 4) gross intraventricular conduction defects 5) cardiac arrest with irregular waves of low voltage Useful therapeutic measures include the attempt to restore electrolyte balance by intravenous injection of hypertonic (3 per cent) NaCl solution, or of hypertonic (25 per cent) glucose solution containing one unit regular insulin per 2 grams glucose, and more fundamentally the removal of the cause of inadequate renal function Numerous mechanical devices for increasing extrarenal excretion (see page 676) have been used, and may be lifesaving if the renal damage is not irreversible

It is difficult to provoke K^+ intoxication in the human subject whose *renal function* is normal Where renal function is questionable it is wise to avoid the use of potassium compounds as drugs, or the use of foods with high K^+ content The manifestations of K^+ intoxication appear to be the result of increased K^+ concentration in plasma and presumably in extracellular fluid, and not to increased K^+ within the cells of the heart In the experimental animal one can increase cellular K^+ by large injections of K^+ salts If water is available, the intracellular levels are quickly restored to normal A more stable increase in cellular K^+ occurs in the advanced stages of experimental adrenal insufficiency in rats extracellular K^+ is also elevated (16)

CHLORIDE

The 70 kgm adult contains about 3 equivalents or 110 grams of Cl^-, no significant portion of which is in *the cells*, or in any form of organic com

bination It is chiefly in the extracellular fluid the red cells of the blood are rich in chloride in comparison to tissue cells their Cl^- content is 77 to 91 mEq per liter The Cl^- of venous blood plasma is 99 to 105 mEq per liter with a mean of 102 mEq , values for arterial plasma are consistently 3 mEq higher—this is the chloride shift, a significant mechanism in CO_2 transport which will be explained in the next chapter The Cl^- content of whole blood depends upon the proportions of cells and plasma plasma or serum analyses for Cl^- may be clinically useful whole blood Cl^- is not

The daily turnover of Cl^- is even more variable than that of Na^+ The chief determinant is the NaCl of the diet which varies from negligible amounts in the rice diet up to 15 grams daily for a heavy salt eater, expressed as Cl^- this would be 9 grams or 330 mEq In the absence of sweating, normal excretion of Cl^- is almost entirely in the urine the daily fecal output is only about 3 mEq

The chloride of extracellular fluids (table 35) approximates the concentration predicted by the Gibbs Donnan equilibrium In the cerebrospinal fluid the Cl^- content is 119 to 128 mEq per liter which is higher than the predicted value With pathological increase of protein content as in tuberculous meningitis, the spinal fluid Cl^- decreases

Bromide

In the blood of dogs or men on an ordinary diet there is almost always a detectable amount of bromide the concentration is 0 1 mEq per liter or less This ion serves no known function in the body Its presence becomes medically significant only when the concentration is considerably increased When a bromide is taken in excessive doses or over a prolonged period of time for its sedative effect its concentration in the blood plasma may reach levels of from 10 to 20 mEq per liter At these levels impairment of the patient's mental functions, particularly memory and concentration, is likely to appear often accompanied by a spotty, purplish discoloration of the skin with elevation very similar to the papules of ordinary acne With even more enthusiastic use of the drug still higher concentrations of circulating bromide may be achieved, as an accompaniment of which the patient shows definite psychotic changes including disorientation lethargy, and confusion There is an unpredictable effect upon reflexes usually shown by disturbances of gait and co-ordination Recovery from such bromide intoxication is usually spontaneous within a few weeks if *the drug is no* longer taken The distribution of bromide in the body *is identical with* that of chloride It will be recalled that bromide *is commonly used to* measure the volume of the extracellular fluid space *Excretion of bromide* and recovery from bromide intoxication *can be accelerated by the* administration of sodium or ammonium chloride (12)

the older units the mean value is 3 5 mgm per 100 ml, with a standard deviation of 0 38 mgm (50)

The concentration in the plasma of children is significantly higher New born infants have almost twice the adult value for plasma inorganic phosphate and from ages 3 to 11, normal levels of children exceed the adult level by about 50 per cent (76) A value which would be normal for a healthy adult would be significantly low in a child The practice of vitamin D supplementation in infant feeding has made it unnecessary to continue previously established differentiations in plasma inorganic phosphate levels between breast fed and bottle fed babies and differences according to latitude and seasons In active rickets the inorganic phosphate usually falls to half the normal infant level or less—a value of 0 6 mgm per 100 ml has been observed—and the alkaline phosphatase activity increases up to four times the normal value The phosphatase increase is often the earliest chemical change in rickets and the last to return to normal under treatment Human rickets is usually the result of vitamin D deficiency (see page 712), often complicated by other deficiencies Experimentally in animals, rickets can be produced by the single deficiency of calcium or phosphate or vitamin D Experimental rickets produced by calcium deficiency is highly atypical and more comparable to osteomalacia If phosphate in the experimental diet is deficient rather typical rickets appears even though the vitamin D intake is adequate

Lower values of plasma inorganic phosphate are characteristically observed in uncomplicated primary hyperparathyroidism and in diseases involving failure of phosphate absorption Increases of plasma inorganic phosphate occur in hypoparathyroidism and in renal disease or urinary obstruction

The inorganic phosphate of the blood must not be considered as inert or as purely a waste product It is derived by hydrolysis of phosphate groups from foodstuffs or metabolites Phosphate ions from plasma may diffuse freely into interstitial fluid and thence may enter cells becoming available for the numerous phosphorylations involved in growth and metabolism Such significant structural substances as nucleoproteins and phospholipids are synthesized in the cell with phosphate ion as a necessary raw material Tracer studies have shown rapid incorporation of phosphate ion into phospholipids Phosphate ion is significantly concerned in acid base regulation which will be dealt with in the next chapter

The inorganic phosphate is of such clinical importance that the other forms of phosphate occurring in the blood are often forgotten Their significance is physiological rather than clinical We find in plasma about 0 5 mgm of ester P and about 8 mgm of phospholipid P per 100 ml Red cells contain about 50 mgm of ester P and about 15 mgm of phospholipid

P per 100 ml The colorimetric method of Fiske and Subbarow (31) is suitable for the measurement of all fractions of phosphate in blood and urine

Excretion of Phosphate

Like calcium, phosphate is excreted both in urine and feces The distribution between the two excretory routes is highly irregular in the normal person with the greater part usually in the feces The urinary excretion is chiefly as inorganic phosphate with only about one per cent in the form of esters or other organic compounds (85)

Parathyroid Activity and the Bone Minerals

The first effect noted when parathyroid hormone is given to a parathyroid deficient patient is an increase in the output of urinary inorganic phosphate There is associated increase of urinary volume with increased chloride output The water loss is greater than the salt loss the patient becomes thirsty This sequence starts within an hour after an adequate dose of parathyroid hormone

Within eight hours a fall in blood plasma inorganic phosphate occurs which is quite abrupt At about this same time a slower increase in blood plasma Ca^{++} begins and continues for several hours The final change, increase in urinary Ca^{++}, is delayed until after the plasma Ca^{++} has noticeably risen

These same end results are found in the hyperparathyroid patient—excessive excretion of both Ca^{++} and phosphate in the urine, diminished inorganic phosphate and increased Ca^{++} in the blood plasma The continued loss of Ca^{++} and phosphate constitutes a drain upon the skeletal stores Eventually decalcification reaches a point where areas of bone salt loss may be seen by x ray, the areas may be local and sharply defined, or may be diffuse and visible only as a general loss of density Hyperparathyroid patients are particularly subject to phosphatic calculi in the urinary tract Hyperparathyroidism is the result of tumor (adenoma or carcinoma) or hypertrophy involving parathyroid tissue The disease is not rare, but is sufficiently uncommon to arouse considerable interest when a case is discovered on a hospital service The diagnosis is not as simple as this brief outline implies The treatment is usually by surgery For serious study of this disease an excellent clinical summary and bibliography is available (1)

There is no disagreement among experts concerning the facts of parathyroid action as outlined It has not been definitely established whether the major function in the human is stimulation of renal excretion—particularly of phosphate ion—or a stimulation of bone-salt solution The

clinical observations seem to favor the renal mechanism Experimen with animals indicate that bone salt is dissolved even when renal actic has been eliminated by nephrectomy The authors of the monograph cite (1) are outspokenly in favor of the renal mechanism but give fair co sideration to the experimental evidence which prevents its acceptance a complete explanation of parathyroid effects We are at present in a positio where we must accept the idea of both a renal action and a bone-dissolvin action of parathyroid hormone, without insisting that either is the caus of the other It is certain that the two actions vary in their importance i different species, the renal action is most significant in the human and wer it not for experiments on dogs it might be accepted as a complet explanation

Parathyroid deficiency in the human is usually the result of actual o functional loss of the glands during or after surgery Rarely the gland degenerate without apparent cause The signs are the reverse of those described as induced by the hormone Blood phosphate is increased, the Ca^{++} depressed so that tetany is the most threatening symptom, urine volumes are low, with diminished excretion of phosphate and other electrolytes

Comparison of parathyroid excess with parathyroid deficiency is the most striking example of the reciprocal relationship between Ca^{++} and phosphate concentrations in plasma Presumably on account of a solubility product relationship with the calcium phosphate or apatite in the bone, increase of one of these ions leads to a decrease of the other This relation ship in plasma can not be stated mathematically with precision, probably because the changes occur through the medium of the interstitial fluid of bone samples of which can not be obtained for analysis

Magnesium

It is proper to consider magnesium among the bone minerals since more of the magnesium of the body is concentrated in the skeleton than in the remaining soft tissue The total magnesium content of the body is about 21 grams or seven eighths of an equivalent The total skeleton contains about 11 grams of magnesium, the muscles contain about 6 grams, with the remainder divided among the other tissues and body fluids Since bone ash contains less than one per cent of magnesium as compared to over 35 per cent of calcium, its relative quantitative importance is obviously minor Its function as an enzyme activator is of much greater physiological sig nificance Certain peptidases and phosphatases require Mg^{++} for maximal activity Another significant enzymatic effect of Mg^{++} occurs in muscle (67) When ATP is added to solutions of actomyosin there is first a de

crease in viscosity which later is slowly reversed The first response is not inhibited by Mg^{++}, *this response does not appear to depend upon the* enzymatic activity associated with myosin The later increase in viscosity is explained by the ATP ase action of myosin This second phase is inhibited by Mg^{++} There is a probability, not yet fully proven, that the first non-enzymic phase is accelerated by Mg^{++} The net effect of increased Mg^{++} concentration on muscle is blockade of neuromuscular transmission (47) The reflexes of decerebrate or amytalized cats disappeared in a different sequence after the injection of magnesium salts from that observed after treatment with volatile anesthetics or with barbiturates In addition to the effect upon muscle there is a direct depressant action upon the central nervous system Although it is not common practice, surgical anesthesia can be induced in man by intravenous injection of magnesium salts Although epsom salt has a venerable therapeutic history its use is not entirely without danger of magnesium poisoning particularly in young children The danger also exists when epsom salt is given in high concentration as an enema (25) The effects upon nerve and muscle functions manifest themselves in magnesium intoxication as coma and flaccid paralysis

The concentration of total Mg^{++} in human blood serum is 1 8 mEq per liter with a standard deviation of 0 12 mEq (85, 48) Analytical methods are described in the papers cited Measurements of total serum magnesium are not particularly rewarding in clinical study except where magnesium poisoning may be expected In one case of coma and flaccid paralysis following an epsom salt enema the serum total Mg^{++} three hours after the enema was 17 mEq per liter (25) In more commonplace clinical situations fluctuations in serum Mg^{++} are relatively small The serum concentration may rise in nephritic patients up to about double the normal value (85) Between 17 and 31 per cent of the normal serum Mg^{++} is bound and not ultrafiltrable The percentage of bound Mg^{++} is increased in hyperthyroidism (19) This bound portion of magnesium disappears in myxedemic patients

The problem of specific isolated magnesium deficiency does not arise in human nutrition Any diet composed of natural foodstuffs and meeting the minimum of other requirements is bound to be adequate in magnesium Intakes as low as 0 22 grams per day yield a positive Mg^{++} balance in the normal The excretion of magnesium, like that of calcium is divided between urinary and fecal channels The average 24-hour urinary excretion of magnesium lies between 8 and 9 mEq The variation in this figure is very considerable For a detailed quantitative study of magnesium retention and excretion under diverse conditions, the reports of Tibbetts and Aub (81) should be consulted

Fluoride

The property which sets fluoride in a different class from the other ions of the body is its extreme toxicity Fatal poisoning has resulted from the ingestion of as little as 0 2 grams of sodium fluosilicate (37), violent painful spastic muscular contractions alternate with periods of flaccid palsy Death is from paralysis of the respiratory muscles Degenerative changes are found *post mortem* in the cells of kidney, liver, and gastrointestinal mucosa Similar poisoning has been reported with somewhat larger doses of simple fluorides Fluoride in toxic concentrations specifically inhibits anaerobic glycolysis (Chapter 12) This effect alone is sufficient to explain its acute toxicity, but does not account for the curious results of chronic ingestion of sublethal doses

If the drinking water of a community contains 1 5 mgm of fluoride per liter, 10 per cent or more of the children using the water during the period of tooth development will show a mild degree of hypoplasia of dental structure called *mottled enamel* With 6 mgm fluoride per liter of drinking water the incidence approaches 100 per cent and intensity of mottling is maximal With 1 0 mgm of fluoride per liter of drinking water, mottled enamel does not occur but the number of cavities in the permanent teeth of the school children (age 12 to 14) of the community is approximately one third of the number of cavities in similar groups from low fluoride (less than 0 2 mgm per liter) communities (63) Increase of fluoride to 2 0 mgm per liter of drinking water produces mottled enamel but without further significant decrease in caries The increased resistance to decay is probably the result of actual alteration of enamel structure by incorporation of fluoride If the intake of fluoride is long maintained at a level of 0 2 mgm per kgm body weight per day *osteosclerosis* or abnormal calcification of periosteum endosteum, and ligaments is likely to occur This has been noted chiefly among workers exposed to the dust of fluoride minerals

There is in normal blood somewhat less than 0 1 mgm fluoride per 100 ml, and a normal urinary content of about 1 mgm per liter (37) In mottled enamel areas the urinary fluoride averages 3 mgm per liter Fluoride is normally concentrated in bones and teeth the amount found is quite variable, being usually between 0 02 and 0 05 per cent in *dry defatted* bone and below 0 03 per cent in teeth Most studies indicate a lower fluoride content of carious teeth compared to sound teeth, but the differences have not always been statistically significant Bone will take up fluoride *in vitro* from very dilute solutions Conversion of bone salt to fluor apatite has been demonstrated in fossil bones

Since the only demonstrable effect of fluoride deficiency is increased

susceptibility to dental decay, the designation of fluoride as an essential dietary component is doubtful Several American cities (Grand Rapids and Midland Michigan Newburgh New York Sheboygan and Madison Wisconsin Evanston Illinois Ottawa, Kansas Marshall Texas) and one Canadian city (Brantford Ontario) have raised the fluoride content of public water supplies to one mgm per liter which is optimal in caries prevention but below the level of mottled enamel production

THE METABOLIC MINERALS

Certain ions are indispensable to the metabolic machinery in that they form compounds necessary to carry out definite operations Usually they are part of individual enzymes or hormones or vitamins some function in a manner not yet fully understood

Iron

The most significant iron compound of living organisms is *heme*, which in most forms of life is a component of important oxidizing enzymes such as the cytochromes catalase and peroxidase In vertebrates, in earth worms and in the root nodules formed by nitrogen fixing bacteria is also found hemoglobin Its function in bacterial nitrogen fixation is not clear In those animals which possess it it is a carrier of oxygen and indirectly of carbon dioxide Its structure has been explained in Chapter 4, its respiratory function will be described in the chapter immediately to follow

To revert to the familiar 70 kgm average adult male we find that each liter of his blood contains about 500 mgm of iron in the form of hemoglobin His blood volume will be a little over 5 liters, and allowing for immature red cells in the bone marrow and non-circulating red cells in the spleen he will have about 3 grams of iron in the form of blood hemoglobin To gain an idea of the distribution in the human body of iron in other forms we are forced to draw an analogy from the distribution in the dog (41) If the proportions are similar in man, there will be about 0 4 grams of iron in the form of muscle hemoglobin and about 0 8 grams as functional tissue iron involving such compounds as cytochromes and catalase About 1 1 grams of iron will be in storage the greater part of it as *ferritin* in liver, spleen and bone marrow Ferritin is a protein which can contain up to 23 per cent ferric iron Its recognized function is that of a storage form of ferric iron in equilibrium with the Fe^{+++} of the blood plasma

About one per cent per day of the red cell hemoglobin iron is released by degradation of hemoglobin to bile pigment This iron is not excreted, but is retained for hemoglobin formation In health red cell formation keeps pace with red cell destruction During the period of functional life of the red cell there is no exchange of hemoglobin iron with plasma iron (29)

The Absorption of Iron

The anemic dog is a highly satisfactory test animal for the study of iron absorption If iron compounds are injected intravenously into such a dog the iron will be utilized practically quantitatively for the formation of hemoglobin If iron compounds are given by mouth to such a dog the amount of hemoglobin formed is a direct measure of the iron actually absorbed If radioactive iron is used as a tracer, it can be detected in the circulating red cells 4 hours after it is fed Complete conversion of absorbed radioactive iron into hemoglobin occurs within a week Normal non anemic dogs do not absorb iron and convert it to hemoglobin with any such effectiveness Since iron excretion has been shown to be negligible, it appears that the amount of iron in the body is regulated through the mechanism of absorption

The only form of iron which is absorbed from the gastrointestinal tract in nutritionally significant quantities is the Fe^{++} ion Ferrous and ferric hydroxides of foodstuffs are converted to salts and dissolved by the HCl of the gastric juice In the acid medium of the stomach, ferric ions are reduced to ferrous with the simultaneous oxidation of ascorbic acid and other reducing agents present in foodstuffs In the dog significant absorption of ferrous salts occurs from the stomach There is physiological evidence for the absorption of ferrous salts from the entire gastrointestinal tract from the stomach on There are undoubtedly species differences in the anatomical location of the site of optimal iron absorption

Iron which is combined in heme or heme derivatives is not available for absorption and is not easily made available in the digestive tract No specific mechanism has been discovered for the digestive liberation of iron from heme An unpredictable and irregular amount of iron is so liberated by the action of micro-organisms Since bacterial action in the gastrointestinal tract is absent or minimal in the stomach and upper intestine, it becomes clear that heme compounds are not reliable sources of nutritional iron This has been borne out by clinical experience—treatment of iron deficiency anemia with foods rich in heme combined iron is highly unsatisfactory compared with the dramatic improvement following the use of *simple* ferrous salts by mouth

The rate of absorption of ferrous iron from the gastrointestinal tract

the ferritin concentration of intestinal mucosal cells increases

cellular ferritin concentrations absorption of iron is blocked The block is slowly removed as the iron of stored ferritin leaves the cell and enters the

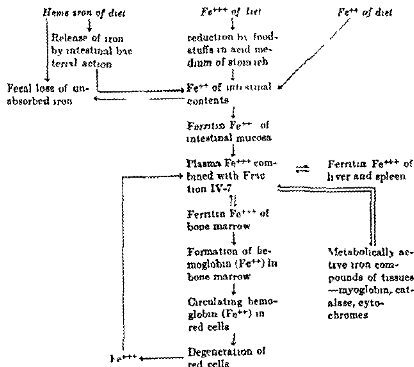
plasma (30) Deficiency in the B-vitamin *pyridoxine* leads to increased iron absorption in experimental animals and an increase in total body iron

Blood Plasma Iron

Iron is transported in the plasma in the form of Fe^{+++} attached to the metal combining globulin (Fraction IV 7) Plasma iron can have its origin

TABLE 36

Pathways, equilibria, and valence changes in iron metabolism



from ferritin of the intestine or any of the storage organs, or it may be liberated directly by the breakdown of blood pigment into bile pigment The mean normal blood plasma iron is 1 05 mgm per liter of plasma, with a standard deviation of 0.33 mgm (8) The iron of the plasma of patients with untreated pernicious anemia or with hemolytic anemia is often elevated, the value is decreased in iron deficiency, in the anemia of infection there is usually a decrease of plasma iron The low concentrations of iron in blood plasma make it necessary to use a very sensitive reagent for its

estimation which is unusually done colorimetrically (2) Iron is removed from plasma by all metabolically active tissues particularly by the bone marrow for hemoglobin formation and by the liver and spleen for storage as ferritin

Excretion of Iron

The body is remarkably conservative in regard to iron A minute amount is excreted in the bile subject to possible reabsorption in the intestine Normal cell free urine contains no iron (59) There is a regular and continuous desquamation of cells which, of course contain iron Such desquamation occurs from the surface of the skin and from the urinary and intestinal tract The rate of such iron loss from the body is so insignificant that nutritional iron deficiency in the adult male is extremely uncommon In women the menstrual loss is about 300 mg of iron a year A loss of blood equivalent to 400 mgm or more of iron may be expected with each childbirth Hemorrhage from accident or disease may, of course impose an iron loss upon any person at any time

Iron Deficiency

In spite of the very small rate of loss of iron from the body under normal circumstances iron deficiency anemia is one of the commonest deficiency diseases in clinical practice The causes of iron deficiency are seldom purely

hemorrhoids can produce an iron deficiency just as effectively as a major hemorrhage Adolescent girls are likely to be deficient in iron on account of the combined effect of the menarche and the high iron requirement for growth at that age In general women are more frequently iron-deficient than men on account of the menstrual loss losses of blood at childbirth and the contribution during pregnancy of about 350 mgm of iron to the fetus

The characteristic change in the blood in iron deficiency anemia is a decrease in the hemoglobin content of the red blood discs There is also a decrease in their size and a less notable decrease in their number The bone marrow shows histological evidence of overactivity with abundant normoblasts The characteristic physical sign is pallor the characteristic symptom fatigue The treatment is by oral administration of ferrous salts in small but frequent doses—small to avoid gastrointestinal irritation and frequent to maintain absorption The toxicity of injected iron salts sets a strict limitation on the use of the intravenous route for iron therapy (70) In severe cases transfusions of whole blood or of red cells are given Pre-

vention is by minimizing blood losses and by adequate intake of iron containing foods, supplemented by ferrous salts during pregnancy and in other situations where iron losses are predictable

Iron Requirements

The allowances stipulated by the National Research Council (see table 37) are generous in regard to iron Since there is always some doubt about the absorbability of iron in foodstuffs it is probably advantageous to allow a wide margin of safety Hahn (41) estimates from uptake experiments with radioactive iron that, even for the period of most rapid growth, 5 mgm per day of absorbable iron is adequate The actual requirements

TABLE 37

Recommended daily dietary allowances of iron

	Iron (mgm)
Man (70 kgm)	12
Woman (56 kgm)	12
Pregnancy (latter half)	15
Lactation	15
Children up to 12 years	
Under 1	6
1 3	7
4 6	8
7–9	10
10–12	12
Children over 12 years	15

for growth, menstruation, and pregnancy have been calculated (45) and fall well under Hahn's estimate

The newborn infant has a store of about 60 mgm of iron in the liver, provided the mother had an adequate diet During the first six months of his life, the infant is likely to live on an iron-deficient diet since the iron content of milk is nutritionally negligible At six months the iron of the liver of an exclusively milk fed infant has decreased to about 15 mgm, 15 mgm have gone into hemoglobin formation, yielding 13 2 grams of hemoglobin More significantly, the normal child is born with a very high hemoglobin content of the blood, 22 grams per 100 ml, which decreases by half during the first two months This decrease is partly by dilution and partly by hemoglobin destruction, the iron from hemoglobin destruction is available for the building of new hemoglobin

At about six months the iron needs of the infant become critical If there has been no significant iron intake, the reserves stored before birth

have been fully utilized It is therefore important that iron rich foods should be included in the diet as early as they can be tolerated

Copper

It is well established that copper is required by rats for adequate synthesis of hemoglobin although of course copper is not a part of the hemoglobin molecule The suggestion has been made that copper is necessary for the formation of cytochrome A and cytochrome oxidase and that these enzymes are necessary in the normal formation of the hemoglobin of red cells A catalytic action of copper in hemoglobin synthesis has been demonstrated in the human infant (49) Copper has also been shown to have a catalytic effect in the development of melanin and possibly other mammalian pigments (32) Copper deficiency can be induced in mammals and leads to abnormalities of pigmentation No state of true copper deficiency has ever been demonstrated in the human using as a criterion actually depressed values of tissue copper

The adult human body contains between 100 and 150 mgm copper liver kidney and brain have the highest concentrations Blood plasma contains about one mgm of copper per liter This value increases gradually during pregnancy is approximately doubled near term and returns to normal within two months after delivery (26) The fetus stores copper particularly in the liver Human red cells contain about 0 7 mgm of copper per liter Certain copper containing proteins have been identified in blood plasma and are summarized by Cartwright (64) The function of these copper proteins has not been established The iron binding protein Fraction IV 7 can unite reversibly with copper as with iron and may function in copper transport Balance experiments indicate that the copper requirement of the average adult is about 2 mgm a day which is fulfilled by almost any conceivable diet The use of copper salts in therapy has had periods of popularity in the past but at present appears irrational as far as human medicine is concerned

Cobalt

Cobalt as a component of vitamin B_{12} (see page 698) is a necessary micronutrient in the human dietary The body utilizes cobalt as far as we know only in the form of vitamin B_{12} or closely related compounds Intestinal synthesis of this vitamin by bacterial action is however a significant source and presumably cobalt taken in other forms is in part incorporated into vitamin B_{12} The function of the vitamin is in the maturation of erythrocytes Cobalt other than as vitamin B_{12} has so far found little rational use in the therapy of human diseases although certain anemias in experimental and domestic animals have been attributed to

cobalt deficiency and have been alleviated by cobalt The human requirement has not been definitely established but is certainly very small, less than a microgram a day

Zinc

Zinc is present in all human organs in amounts varying roughly from 20 to 180 micrograms per gram of wet tissue Whole human blood contains 8 8 ± 2 0 and plasma contains 3 0 ± 1 6 micrograms per ml (82) The white cells of the blood contain about 25 times as much zinc (per cell) as the red cells The function of the zinc of the white cells is not known, but that in the red cells is accounted for by *carbonic anhydrase* (see page 623) an enzyme which contains zinc No figure for the daily human requirement of zinc has been determined Average human diets contain 10 to 15 mgm per day one mgm or less is excreted in the urine and the remainder in the feces Growth failure hair loss and skin eruptions have been noted in rats brought up on zinc-deficient rations Some suggestions of zinc deficiency in the human have arisen in connection with beriberi and other diseases *involving chronic deficiency of several known food factors* (82) *but no specific zinc deficiency diseases has been described in man*

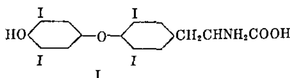
Carbonic anhydrase is the only compound in which zinc is known to be necessary for its physiological function Certain other enzymes including alkaline phosphatase and zymohexase are reported to be activated by zinc and the hormone insulin is most conveniently crystallized as a zinc salt In none of these cases has it been demonstrated that the zinc is a part of the substance as it occurs in the body as in carbonic anhydrase

Iodine

In 1895 Baumann found iodine in thyroid tissue in organic combination and surmised that the physiological potency of thyroid was related to the iodine content Oswald in 1901, isolated an iodine-containing protein thyroglobulin from thyroid In 1916 Kendall obtained thyroxine an iodine containing amino acid component of thyroglobulin In 1926 Harington devised methods for improving the yield and proposed the accepted structural formula In 1927 Harington and Barger proved the formula by synthesis (formula I)

Thyroxine exists in dextro and levo forms The levo isomer is effective in the treatment of myxedema and is the form which is present in the thyroid All of the iodine of thyroid is not present in the form of thyroxine about 5 per cent is present as iodide ion (58) Monoiodotyrosine demonstrable by paper chromatography, is a normal component of the thyroid gland of the rat Of the total iodine of the hydrolysate of rat thyroid approximately 15 per cent is in the form of monoiodotyrosine 30 per cent

diiodotyrosine, and 20 per cent or more thyroxine (80) Iodine is 10 000 times more concentrated in *thyroid than in any other tissue or organ* Aside from this remarkably high concentration in the thyroid gland iodine behaves much like any other halogen as far as distribution throughout extracellular fluid is concerned Following large doses it appears in the gastric juice, replacing chloride It is excreted in the urine Since the time of Baumann we have known that the average concentration of iodine in the thyroid gland is 40 mgm per 100 grams of fresh tissue, this makes the total iodine content of the thyroid about 10 mgm The thyroid takes up iodine from the blood and from perfusion fluid This uptake reaches a maximum *in 10 minutes or less following an increase in the iodine content* of the blood or perfusion fluid For uptake to occur the iodine must be in the form of iodide ion iodine in the form of thyroxine diiodotyrosine or iodate is not specifically concentrated by the thyroid Iodine in other forms is slowly converted to iodide in the blood after which it is absorbed by the thyroid (58) The thyroid becomes saturated with iodine when the



concentration in the gland is increased above the normal by 10 to 20 mgm
' ~ noo pigs rabbits and
fix iodine in the

The use of radioactive iodine has cleared up many problems concerning iodine metabolism in the thyroid gland The average daily human absorption of iodide is 0 05 to 0 1 mgm The use of a radioactive tracer shows that in such dosage 50 per cent of the iodide is in *the thyroid gland* within 48 hours Iodide entering the thyroid quickly attaches to tyrosine forming diiodotyrosine groups The further formation of thyroxine is slower and is mediated by an enzyme system involving peroxidase and possibly xanthine oxidase When radioactive thyroxine and diiodotyrosine are detected in the thyroid they are found in combination as thyroglobulin with about 60 per cent of the iodine in the form of diiodotyrosine The iodine leaving the thyroid and entering the blood is 10 per cent in diiodotyrosine 90 per cent in thyroxine The colloid of the thyroid follicles contains a proteo*lytic enzyme which is present in greater amount* when the thyroid secretion is experimentally stimulated by thyrotropic hormone The release of thyroxine and diiodotyrosine into the blood is considered to be the result of the liberation of these amino acids from thyroglobulin by proteolysis The

presence of the proteolytic enzyme is associated with basophilia of the colloid. Basophilia is characteristic of actively secreting follicles, as opposed to the acidophilia of resting stages or following hypophysectomy.

A daily dose of 0.25 to 0.35 mgm. of thyroxine is required to keep a thyroidectomized patient up to a normal metabolic rate. The iodine equivalent of such a dose of thyroxine would be about 0.2 mgm. Since the daily absorption is less than this, it is apparent that iodine is re-used by the thyroid. Thyroxine is broken down in the blood or tissues, and the liberated iodide returned to the thyroid to be worked over. Thyroxine rapidly disappears after injection, although it is not destroyed by incubation with blood *in vitro*. The liver is active in the destruction and removal of thyroxine. Thyroxine has a greater effect in hepatectomized than in normal animals. Injected diiodotyrosine in small doses is rapidly destroyed in the body, releasing its iodine as iodide.

The iodine of the blood consists of two fractions: (a) inorganic iodide, which is unaffected by thyroid function but increases with administration of iodine compounds, and (b) protein bound iodine, which directly reflects thyroid activity. The inorganic iodide normally varies between 0.5 and 4 micrograms per 100 ml. human plasma (3). The protein bound iodine is normally between 3 and 8 micrograms per 100 ml. plasma; it includes the iodine of circulating thyroxine, and is decreased in hypothyroidism and increased in hyperthyroidism. Urinary excretion of a minute dose of radio-iodide is 37 per cent within 18 hours, the fecal excretion in the same time, 17 per cent. Thyroglobulin is physiologically active upon oral administration, as are iodinated proteins. Since proteins are hydrolyzed in the digestive tract, it is apparent that the hydrolytic products are physiologically active.

Ordinary albumin, casein, and other proteins may be iodinated in such a way that the products are effective in a manner comparable to thyroglobulin. Crystalline thyroxine has been isolated from iodinated proteins of known biological activity (68). The amount of thyroxine isolated was proportional to the biological activity of the proteins.

The enzymatic conversion of iodide ion by the thyroid gland to diiodotyrosine and thyroxine is inhibited in the rat when the plasma level of iodide ion goes above 20 to 35 micrograms per 100 ml. (88). Although the organic binding of iodine ceases, iodide ion is still taken up and stored by the thyroid. Such a mechanism in the human would explain the favorable therapeutic effects of iodine and iodide in hyperthyroidism. Drugs other than iodine which depress thyroid action include thiocyanates, which inhibit the entry of iodine into the thyroid, and particularly thiouracil and its derivatives, which inhibit the enzyme system which converts iodide into thyroxine, hence increase iodine storage. The thyrotrophic hormone of the

anterior pituitary increases the ability of the thyroid to retain iodide, but hypophysectomy does not abolish this ability (58) It is concluded that the thyrotrophic hormone increases the size of thyroid cells but does not modify their iodine fixing function The pituitary thyrotrophic hormone does appear to be necessary, however, for the final conversion into thyroxine There is some evidence for the formation of diiodotyrosine and thyroxine outside the thyroid gland Radioactive diiodotyrosine and thyroxine have been demonstrated in thyroidectomized rats following the administration of radio iodine

The iodine content of soil and foodstuffs is highly variable with geographic distribution Only in foodstuffs of marine origin can iodine be said to be uniformly plentiful The actual requirement of iodine by the human body is small compared to that of other minerals of comparable significance An intake of 0 1 mgm daily is adequate Lack of iodine is *reflected in disturbances of thyroid structure and function* Most commonly this is a simple increase in the size of the thyroid causing no disturbances other than cosmetic and possibly mechanical Less frequently there is degeneration of the thyroid with loss of function There is no unanimity of opinion concerning the question of iodine deficiency as a cause of hyper thyroidism nor of iodine deficiency as a contributory cause of malignancy of the thyroid

Simple goiter is enlargement of the thyroid gland without marked alter ation of its physiological function Its best recognized cause is deficiency of iodine in the diet (21) It is endemic in certain regions where the soil and native foodstuffs are poor in iodine A further cause may be dietary Certain foodstuffs, for example turnips, contain 'anti thyroid factors' which may presumably interfere with the metabolism of iodine This theory has some verification by animal experimentation and may explain why the entire population of goiter belts is not afflicted There are no particular biochemical changes in the body in simple or endemic goiter The signs of hypo- or hyperthyroidism are absent Hypothyroidism may develop in cases of simple goiter Hyperthyroidism following simple goiter or its treatment is uncommon in American experience, although European writers mention the hazards of ' Iodbasedow " Simple goiter can be prevented by assuring an annual intake of 50 mgm of iodine as iodide For a discussion of the medical management of cases of simple goiter see Youmans (90)

REFERENCES

1 Albright, F , and Reifenstein, E C *The Parathyroid Glands and Metabolic Bone Disease* Baltimore, Md , The Williams & Wilkins Co 1948
2 Barkan, G , and Walker, B S Determination of serum iron and pseudohemo globin iron with *o* phenanthroline J Biol Chem , 135 37-42 1940

3 Bassett A M et al Protein bound iodine in blood V Naturally occurring iodine fractions and their chemical behavior Am J M Sc 202 516-527 1941
4 Bevelander G and Johnson P L The histochemical localization of glycogen in the developing tooth J Cell & Comp Physiol 28 129 135 1946
5 Bodansky A Phosphatase studies determination of serum phosphatase Factors influencing accuracy of determination J Biol Chem 101 93 104 1933
6 Brodie B B et al The use of bromide as measure of extracellular fluid J Biol Chem 130 555 563 1939
7 Burch G et al Rates of sodium turnover in normal subjects and in patients with congestive heart failure J Lab & Clin Med 32 1169-1191 1947
8 Cartwright G E et al Studies on free erythrocyte protoporphyrin, plasma iron and plasma copper in normal and anemic subjects Blood 3 501 525, 1948
9 Clark E P and Collip J B A study of the Tisdall method for the determination of blood serum calcium with a suggested modification J Biol Chem 63 461-464 1925
10 Consolazio W V and Talbott J H The determination of total base in biological material by electrodialysis J Biol Chem 132 [illegible] 1940
11 Corcoran A C and Page I H The kidney In Sodeman W A Pathologic Physiology Mechanisms of Disease pp 481 510 Philadelphia W B Saunders 1950
12 Cornbleet T Bromide intoxication treated with ammonium chloride J A M A 146 1116-1119 1951
13 Dallemagne M J The physiology of supporting tissue Ann Rev Physiol 12 101 118 1950
14 Danowski T S The transfer of potassium across the human blood cell membrane J Biol Chem 139 693 705 1941
15 Darnell M C and Walker B S Determination of sodium in biological fluids Indust & Engin Chem Analyt Ed 12 242 244 1940
16 Darrow D C Body fluid physiology The relation of tissue composition to problems of water and electrolyte balance New England J Med 233 91 97 1945
17 Davis H A Shock and Allied Forms of Failure of the Circulation New York Grune and Stratton 1950
18 Dill D B Life Heat and Altitude Cambridge Mass Harvard University Press 1938
19 Dine R F and Lavietes P H Serum magnesium in thyroid disease J Clin Investigation 21 781 786 1942
20 Duncan G G Diseases of Metabolism Chapter XIV Diabetes insipidus Philadelphia W B Saunders 1947
21 Editorial Relation of endemic goiter to lack of iodine Nutrition Rev 6 40-43 1948
22 Elkinton J R Physiology of body fluids In Fulton J F A Textbook of Physiology pp 863 890 Philadelphia W B Saunders 1949
23 Elkinton J R and Winkler A W Transfers of intracellular potassium in experimental dehydration J Clin Investigation 23 93 101, 1944
24 Fankuchen I X ray studies on compounds of biochemical interest Ann Rev Biochem 14 207 224 1945
25 Fawcett F W and Gens J P Magnesium poisoning following an enema of epsom salt solution J A M A 123 1028 1029 1943
26 Fay J et al Studies on free erythrocyte protoporphyrin serum iron serum

iron binding capacity and plasma copper during normal pregnancy J Clin Investigation, 28. 437–491, 1949
27 FELDBERG, W Present views on the mode of action of acetylcholine in the central nervous system Physiol Rev, 25 596–642, 1945
28 FENN, W O The role of potassium in physiological processes Physiol Rev, 20. 377–415, 1940
29 FINCH, C A, et al Iron metabolism erythrocyte iron turnover J Lab & Clin Med, 34: 1480–1490, 1949
30 FISKE C H, AND LOGAN, M A Determination of calcium by alkalimetric titration J Biol Chem, 93. 211–226, 1931
31 FISKE, C H, AND SUBBAROW, Y The colorimetric determination of phosphorus J Biol Chem, 66 375–400, 1925
32 FLESCH, P The role of copper in mammalian pigmentation Proc Soc Exper Biol & Med, 70. 79–83, 1949
33 FOLK, B P, et al Distribution of potassium and sodium between serum and certain extracellular fluids in man J Physiol, 153 381–385, 1948
34 FREEMAN, F, AND CHANG, T S Role of the kidney and of citric acid in production of a transient hypercalcemia following nephrectomy Am J Physiol, 160: 335–340, 1950
35 GAMBLE, J L *Chemical Anatomy, Physiology, and Pathology of Extracellular Fluid* 5th edit Cambridge, Mass, Harvard University Press, 1950
36 GRANICK, S Iron and porphyrin metabolism in relation to the red blood cell Ann New York Acad Sc, 48. 657–681, 1947
37 GREENWOOD, D A Fluoride intoxication Physiol Rev, 20 582–616, 1940
38 GREEP, R O, et al Alkaline phosphatase in odontogenesis and osteogenesis and its histochemical demonstration after demineralization J Am Dent A, 36. 427–442, 1948
39 GUBLER, C J, et al The effect of pyridoxine deficiency on the absorption of iron by the rat *J Biol Chem* 178. 989–996, 1949
40 GUTMAN, A B, AND YU, T F Further studies of the reaction between glyco [illegible] Trans 1st [illegible]
4[illegible] hemoglobin metabolism and the physiology of the erythrocyte Advances M & Biol Physics, 1: 288–319, 1948
42 HAHN, P F, AND WHIPPLE, G H Iron metabolism in experimental anemia "Availability of iron" J Exper Med, 67. 259–265, 1938
43 HASTINGS, A B, et al The ionization of calcium, magnesium, and strontium citrates J Biol Chem, 107. 351–370 1934
44 HASTINGS, A B, et al The distribution of electrolytes between transudates and serum J Gen Physiol, 8 701 711, 1927
45 HEATH C W Iron in nutrition J A M A, 120 366–370, 1942
46 HOAGLAND, H Stress and the adrenal cortex with special reference to potassium metabolism Psychosom Med, 12 142 148, 1950
47 HOFF, H E et al Effects of magnesium on the nervous system in relation to its concentration in serum Am J Physiol, 130 292 297, 1940
48 HOFFMAN, W S A colorimetric method for the determination of serum magnesium based on the hydroxyquinoline precipitation J Biol Chem, 118 37–45 1937

49 HUTCHISON J H The role of copper in iron deficiency anaemia in infancy Quart J Med 7 397–418 1938
50 JELLINEK E M AND LOONEY J M Statistics of some biochemical variables on healthy men in the age range of twenty to forty five years J Biol Chem 128 621–630 1939
51 JOHNSTON F A et al Methods for determining the iron content of milk J Biol Chem, 175 343 347 1948
52 KEHOE R A et al A spectrochemical study of the normal ranges of concentration of certain trace metals in biological materials J Nutrition 19 579–592, 1940
53 KEITH N M AND BURCHELL H B Clinical intoxication with potassium its occurrence in severe renal insufficiency Am J M Sc 217 1 12 1949
54 KEMPNER W Treatment of heart and kidney disease and of hypertensive and arteriosclerotic vascular disease with the rice diet Ann Int Med 31 821–856 1949
55 LADELL W S S The changes in water and chloride distribution during heavy sweating J Physiol 108 440–450 1949
56 LAIDLAW J C Nature of the circulating thyroid hormone Nature 164 927–928, 1949
57 LEAF, A AND COUTER W T Evidence that renal sodium excretion by normal human subjects is regulated by adrenal cortical activity J Clin Investigation 28 1067 1081 1949
58 LEBLOND C P Iodine metabolism Advances M & Biol Physics 1 353–386 1948
59 LINTZEL W Zur Frage des Eisenstoffwechsels ueber das Harneisen Ztschr f Biol 87 157 166 1928
60 LOEB R F AND BENEDICT E M Inorganic sulphates in human blood J Clin Investigation 4 33–36 192?
61 MARINIS T P et al Sodium and potassium determinations in health and disease J Lab & Clin Med 32 1208–1216 1947
62 MARRIOTT H L Water and salt depletion Brit M J 1 245–250 285–290 328 332 1947
63 McCLURE F J Fluorine and other trace elements in nutrition J A M A 139 711 716 1949
64 McELROY W D AND GLASS B *Copper Metabolism* Baltimore Md Johns Hopkins Press 1950
65 McLEAN F C AND HASTINGS A B Clinical estimation and significance of calcium ion concentration in the blood Am J M Sc, 189 601–613 1935
66 MITCHELL H H et al The chemical composition of the adult human body and its bearing on the biochemistry of growth J Biol Chem 158 625–637 1945
67 MOMMAERTS W F H M The reaction between actomyosin and adenosine triphosphate J Gen Physiol 31 361 375 1938
68 PITT RIVERS R The chemical assay of biologically active iodinated proteins isolation of thyroxine Biochim et Biophys Acta 3 648–654 1949
69 PUDENZ R H et al The role of potassium in familial periodic paralysis J A M A 111 2253 2258 1938
70 RAMSEY, A S *Intravenous iron in the treatment of anaemia* Brit M J 1 1109–1112 1950
71 ROURKE G M et al Case records of the Massachusetts General Hospital New England J Med 234 21 28 1946

72 SHEPPARD, C W New developments in potassium and cell physiolog
1950 Science, 114: 85–91, 1951
73 SHERMAN, H C *Calcium and Phosphorus in Foods and Nutrition* Ne
Columbia University Press, 1947
74 SOBERMAN, R, *et al* The use of antipyrine in the measurement of to
water in man J Biol Chem, 179: 31–42, 1949
75 SOLOFF, L A, AND ZATUCHNI, J Syndrome of salt depletion J A M
1136–1139, 1949
76 STEARNS, G, AND WARWEG, E The partition of phosphorus in whole b
serum, the serum calcium and plasma phosphatase from birth to m
J Biol Chem, 102. 749–765, 1933
77 STRAUSS, M B, *et al* The effect of alcohol on the renal excretion of w
electrolyte J Clin Investigation, 29. 1053–1058, 1950
78 TARAIL, R Relation of abnormalities in concentration of serum pota
electrocardiographic disturbances Am J Med, 5. 828–837, 1948
79 TARAIL, R, AND ELKINTON, J R Potassium deficiency and the role of th
in its production J Clin Investigation, 28: 99–113, 1949
80 TALBOG, A, *et al* The monoiodotyrosine content of the thyroid gland
Chem 184: 83–97, 1950
81

82
zinc content of erythrocytes in normal, in anemic, and other path
conditions Blood 4 467–478, 1949
83 VERNEY, E B Absorption and excretion of water, antidiuretic hormone
2: 739, 781, 1946
84 WALKER, B S, AND WALKER, E W The organic fraction of urinary phos
J Lab & Clin Med, 18: 164 166 1932
85 WALKER, B S, AND WALKER E W Normal *magnesium metabolism*
significant disturbances J Lab & Clin Med, 21: 713 721, 1936
86 WIGGERS, C J *Physiology of Shock* New York, The Commonwealth Fun
87 WOLF, A V *The dehydrating effect of continuously administered wa*
Physiol 143 567–571 1945
88

89
biological fluids Their estimation by flame photometry M J Aust
(37th year) 821 835, 1950
90 YOUMANS, J B Mineral deficiencies J A M A, 143: 1252–1259, 1950

CHAPTER 16

Respiration and Acidosis

Each individual cell respires. Aerobic oxidations, which supply the major portion of cellular energy, require molecular oxygen as the ultimate hydrogen acceptor. Carbon dioxide is produced by the decarboxylation of organic acid metabolites, as in the citric acid cycle. Internal or cellular respiration is the gas exchange of individual cells: their uptake of oxygen and output of carbon dioxide. Blood and extracellular fluid transfer oxygen and carbon dioxide between cells and pulmonary alveolar capillaries. This can be called intermediary respiration. In the alveoli of the lung, gas exchange occurs between blood and alveolar air. This, together with pulmonary ventilation—the physical movement of air in and out of the lungs, can be designated as external respiration.

THE GAS LAWS

At a constant temperature the product of the pressure and the volume of a confined sample of a perfect gas is constant. If the temperature is varied, the pressure-volume product varies in proportion to the absolute temperature. These relationships can be summarized:

$$PV = nRT$$

where P is pressure, V is volume, n is number of mols of gas, T is the absolute temperature, and R is the gas constant, which must be expressed in units conforming to the units used in measuring pressure and volume. If pressure is given in atmospheres and volume in liters, the value of R is .082 liter-atmospheres.

This perfect gas equation is derived from the kinetic theory of gases and assumes that the individual gas molecules occupy no significant portion of the total volume and that there is no attraction nor repulsion between individual gas molecules. These assumptions do not hold for any actual gas, therefore this is an approximate, and not an exact, physical law. The deviations from the perfect gas law are greatest when (a) the gas is near its liquefying point, or (b) the gas is under high pressure. A more exact but more troublesome form of the perfect gas equation involves corrections for molec

ular volume and for intermolecular forces—the van der Waals corrections The physiologically significant gases closely approximate the behavior of a perfect gas at physiological temperatures, hence van der Waals corrections are seldom necessary in physiological studies For the application and derivation of the van der Waals corrections and the derivation of the perfect gas equation, the interested student may consult texts of physical chemistry

The perfect gas equation applies also to a mixture of gases so that at constant volume and temperature the fraction of the total pressure exerted by one component is equal to its *mol fraction* By mol fraction is meant the mols of the component in question divided by the total mols of all components of the gas mixture If the pressure is constant, the fraction of the total volume occupied by one component is equal to its mol fraction To summarize, assuming a mixture of gases under constant volume, pressure, and temperature, the mol fraction of any single gas equals its pressure fraction and equals its volume fraction *Partial pressure* is the actual pressure exerted by a single component, and equals total pressure multiplied by mol fraction Note that the percentage by volume of a single component equals 100 multiplied by mol fraction These relationships can be exemplified by a consideration of outdoor air and of alveolar air

OUTDOOR AIR

If the water vapor is removed from a sample of outdoor air, and the sample is then analyzed its composition will be found to be as shown in the first two columns of table 38 These values are constant

If the dry outdoor air is at the same pressure as that of the atmosphere which has a mean pressure at sea level of 760 mm Hg the partial pressure exerted at mean atmospheric pressure by each component will be found by multiplying its mol fraction by 760 mm Hg These values make up the third column of table 38

The partial pressures of O_2 and CO_2 determine the direction of movement of these respiratory gases in the lungs, body fluids, and tissues Each gas moves with the gradient of partial pressure from regions of high partial pressure to regions of lower partial pressure

The actual partial pressure of oxygen in outdoor air may occasionally be higher than the value given in the table This would occur in dry weather with high barometric pressure More frequently, the actual partial pressure of oxygen is somewhat less than the quoted figure since outdoor air usually contains water vapor which contributes to the total volume and pressure Physiologically, we are more concerned with pressure than with volume since pressure is the motive power of gas exchange in lungs and tissues We therefore calculate the correction for water vapor in terms of pressure The

vapor pressure of water has been experimentally determined over the entire range of temperatures compatible with life If air is saturated with water vapor, the barometric pressure is corrected by subtracting the known vapor pressure of water (often called aqueous vapor tension') at the observed temperature from the observed barometric pressure The actual partial pressure of oxygen or any other atmospheric gas is then calculated by multiplying its mol fraction by the corrected barometric pressure

If air is neither dry nor saturated with water vapor (this is the usual situation with outdoor air) the vapor pressure of water at the observed temperature must first be multiplied by the fraction of saturation with water vapor This fraction is the *relative humidity*, which is usually stated as a percentage It can be measured by the use of a sling psychrometer or similar instruments measuring the differences between the temperatures indicated by a dry bulb and a wet bulb thermometer

TABLE 38

Composition of outdoor air

	PER CENT BY VOLUME	MOL FRACTION	PARTIAL PRESSURE
			mm Hg
Oxygen	20 94	0 2094	159 1
Carbon dioxide	0 04	0 0004	0 3
Nitrogen and rare gases	79 02	0 7902	600 6

The corrections just described are necessary to establish the exact value of the partial pressure or mol fraction or partial volume of oxygen or carbon dioxide in outdoor air In the majority of quantitative respiration experiments it is not necessary to establish this value The volume of dry air inspired during a given time can ordinarily be calculated from the nitrogen of the expired air collected during that time This calculation depends upon the fact that the dissolved nitrogen of the blood and tissue fluids is in saturation *equilibrium with the nitrogen of the air and there is neither gain* nor loss of nitrogen by the body during respiration under normal partial pressures of nitrogen

During respiration under conditions of greatly increased air pressure, as in the caissons used in tunneling under bodies of water, the tissues and body fluids dissolve more nitrogen than at ordinary atmospheric pressures, following Henry's Law—at constant temperature the solubility of a gas in a liquid is proportional to the partial pressure of the gas Since molecular nitrogen is metabolically inert, such an increase in dissolved nitrogen produces no disturbance The danger of high air pressures lies in too rapid a return to normal pressure Bubbles of gas, chiefly nitrogen, form in the

tissues causing local or referred pain Nitrogen is more soluble in fatty than in non fatty tissue, which accounts for the particular tendency for such bubbles to form in fascial planes and in the fat of the popliteal region *Aeroembolism*, or the formation of bubbles within the blood stream is less likely to occur, since the arterial blood is in equilibrium with alveolar and atmospheric air during decompression. Aeroembolism is potentially fatal, and may occur with extremely rapid decompression, or as a result of local spasm of blood vessels or their compression by extravascular bubbles The attacks of localized pain resulting from decompression are known to caisson workers as "the bends" Trouble is avoided by lowering the pressure in stages, each stage decreasing the excess pressure by one half, until the excess pressure is not more than one atmosphere Decompression from one atmosphere can be accomplished safely in one stage A similar but more complex situation develops during rapid ascents in aircraft from ground level to altitudes above 30,000 feet (226 mm Hg barometric pressure), and *during relatively slow ascents above this altitude* Likewise, if a pressurized aircraft cabin is punctured at high altitude, the occupants are rapidly decompressed The management of these difficulties is discussed in the specialized literature of aviation physiology

ALVEOLAR AIR

The air in the alveoli of the lungs differs in composition from the atmospheric air In the first place, it is nearly or fully saturated with water vapor at body temperature In calculations of partial pressures it is usually assumed to be fully saturated Actual measurements indicate a lag in reaching body temperature, full saturation, or both The discrepancy during normal respiration is not more than 2 mm Hg of water vapor pressure During forced breathing the lag may be greater

At 37°C the partial pressure of water vapor at saturation is 47 mm Hg The total pressure of air in the bronchial tree, including the alveoli is identical with the barometric pressure outside *during all phases of normal* pulmonary ventilation Therefore the partial pressure of any single component of the alveolar air equals the mol fraction of that component multiplied by (barometric pressure minus 47) mm Hg

Since the entire volume of air in the lungs is not exchanged with each inspiration and expiration, the air which enters the alveoli is a mixture of freshly inspired air and air which has already exchanged gases with the blood Direct analysis of alveolar air is impossible in the intact human subject No great error is introduced by the assumption that the last portion of air

that of alv

at rest and at sea level show an average oxygen partial pre

mm Hg (4). Analysis of the arterial blood of the same subjects gave an average value of 97.1 mm Hg. An indirect method has been devised (12) for calculating the partial pressures of alveolar CO_2 and O_2 from the partial pressures of CO_2 in arterial blood and of CO_2 and O_2 in the expired air. The results obtained by this method are designated as the "effective" partial pressures and represent a physiological mean pressure. Using this method a greater partial pressure gradient can be demonstrated for oxygen between alveolar air and arterial blood. This difference averages 9 mm Hg at rest and 16.5 mm Hg after achieving a steady state during the performance of work. Lowered oxygen content of inspired air to a simulated altitude of 16,500 feet produced no significant alteration of the gradients. Average values of the partial pressures of the respiratory gases in alveolar air and arterial blood in the resting human subject at sea level may be seen in table 39. A variation of ±7 mm Hg for oxygen and of ± 5 mm Hg for carbon dioxide may occur. The partial pressures of carbon dioxide in alveolar air and arterial blood are shown as identical in the table. Actually the blood shows about 0.5 mm Hg more carbon dioxide than the alveolar air. The slight differences in partial pressure are not so much the result of failure of equilibration in the alveoli as from the fact that all the blood returning from the lungs has not passed through alveolar capillaries.

Increased pulmonary ventilation increases the rate of removal of carbon dioxide from the entire bronchial tree, thus reducing the concentration and therefore the partial pressure of carbon dioxide in the alveolar air. By voluntary forced breathing the alveolar partial pressure of carbon dioxide can be reduced to about 15 mm Hg. By the same maneuver the alveolar partial pressure of oxygen is increased to around 150 mm Hg.

OXYGEN TRANSPORT BY THE BLOOD

Oxygen enters the blood by diffusion through the walls of the alveolar capillaries and is dissolved in the blood plasma. A small portion remains dissolved in the plasma and is thus transported in physical solution. A larger portion leaves the plasma and unites with hemoglobin, the chromoprotein of the red blood discs. Note that all the transported oxygen must first be dissolved in plasma. Referring again to Henry's Law, the amount of oxygen which dissolves in a given volume of plasma is proportional to the partial pressure of oxygen in the gas phase with which the plasma is in equilibrium. For reasons noted in the preceding section, the dissolved oxygen in the blood may not quite reach equilibrium with the oxygen of the alveolar air.

The *absorption coefficient* of oxygen in blood plasma at 37°C. is 0.027. The absorption coefficient is the volume (measured at the standard conditions of 0°C. and one atmosphere) of a specified gas which will be dissolved

figure 21, and the location of the curve depends upon the partial pressure of CO_2 Increasing the CO_2 of the gas mixture at a given partial pressure of oxygen favors the dissociation of oxyhemoglobin into unoxygenated hemoglobin and oxygen This peculiarity of the behavior of oxyhemoglobin makes the unloading of oxygen more complete in the tissue capillaries,

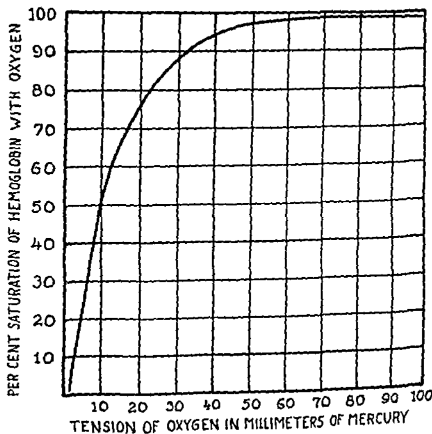


FIG 20 Oxygen saturation curve of hemoglobin

where the partial pressures of CO_2 are high In the lungs, release of CO_2 to the alveolar air takes place, lowering the partial pressure of CO_2 and thereby increasing the ability of the hemoglobin to combine with oxygen at its prevailing partial pressure

A number of physiological adaptations are indicated by the shape of the dissociation curve of oxyhemoglobin It is clear that under physiological conditions 100 per cent saturation of hemoglobin with oxygen is never attained This could be accomplished at partial pressures of oxygen com

parable to those prevailing in outdoor air but such concentrations of oxygen do not occur in the pulmonary alveoli At the partial pressures of oxygen in alveolar air, the saturation of hemoglobin is about 95 per cent At partial pressures of this magnitude, 80 mm Hg or over the curve has distinctly flattened Variations in alveolar oxygen above this figure have

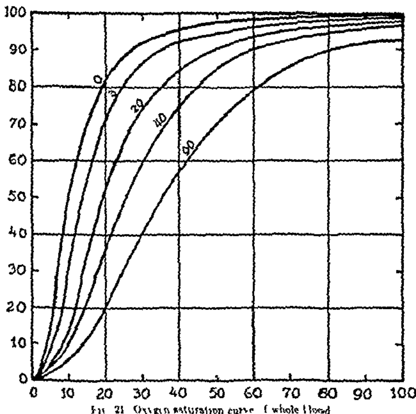


Fig 21 Oxygen saturation curve of whole blood

little effect upon the oxygen content of arterial blood The steep slope of the dissociation curve between partial pressures of 20 and 60 mm Hg corresponds to the difference in oxygen partial pressure between arterial blood and tissues, and permits effective unloading of oxygen in regions where oxygen is deficient Just as full saturation is not attained physiologically complete deoxygenation of the blood does not occur The venous blood of a resting subject is still 60 to 70 per cent saturated with oxygen and with activity this figure may fall to 25 per cent The sigmoid or "S" shape of the

whole blood curve is obviously favorable to oxygenation at partial pressures above 60 mm Hg, and to deoxygenation at partial pressures below 50 mm Hg The tendency to deoxygenation is less in the curve of the pure hemoglobin solution, and is less at temperatures below human body temperature A further advantage of being a warm blooded animal lies in the increased rate of oxygenation and dissociation with temperature At body temperature adjustment of oxygen saturation to altered partial pressure of oxygen occurs in about 0 01 second

The volume of oxygen taken up by the circulating blood in the lungs is normally larger than the volume of carbon dioxide given off The ratio is

$$\frac{\text{volumes } CO_2 \text{ output}}{\text{volumes } O_2 \text{ intake}}$$

the *respiratory quotient* (RQ), and has a value of unity only in the theoretical case where only carbohydrate is being metabolized Examination of the formula for any utilizable carbohydrate will show that it already possesses enough oxygen to take care of the oxidation of its hydrogen to water Additional oxygen is necessary only for the oxidation of the carbon of the molecule to CO_2 With all other foodstuffs, some oxygen is required for the oxidation of hydrogen in addition to that required for the oxidation of carbon Hence for all foodstuffs other than carbohydrate the respiratory quotient is less than unity In the fasting subject, the respiratory quotient is 0 82 ± 0 05, on an ordinary diet the value is about 0 85, decreasing to a limit of 0 7, which value indicates the exclusive metabolism of fat

Although the gaseous nitrogen of the atmosphere is not metabolized by the body, there is a difference in the concentration (usually expressed as a difference in the partial pressure) of nitrogen in expired as compared with inspired air One might be led to believe from the crude figures that the body produced nitrogen gas by the metabolism of nitrogenous foodstuffs This is definitely not so The apparent increase in nitrogen concentration is the result of the greater amount of oxygen uptake compared with carbon dioxide output Only in the hypothetical case where the respiratory quotient is unity, indicating utilization of a purely carbohydrate diet would the nitrogen content and partial pressure be the same in expired as in inspired air

Although nitrogen takes no part in metabolism, a considerable amount is transported in the dissolved state in the blood plasma This amount is proportional to the partial pressure of nitrogen in the alveolar air, and amounts to about 1 5 liters (under standard conditions) in the total body fluids and tissues of an average person

Muscle hemoglobin or *myoglobin* is not identical with blood hemoglobin and has a very different dissociation curve It is still 60 per cent saturated

at oxygen partial pressures of 5 mm Hg It loses its oxygen rapidly at partial pressures below that value Its physiological function is that of a reserve supply of oxygen in those rare situations when the blood reaching the muscle is almost completely deoxygenated

HYPOXIA

Deficiency of oxygen supply to tissues, locally or generally is designated as hypoxia, or if extreme, as anoxia Either may be the result of (a) de- [illegible] deficient delivery [illegible] alls entirely into [illegible] will not be considered in this book Concerning the first category, chemical changes in the blood may be causative or contributing factors in the production of hypoxic states

Failure of the blood to become adequately oxygenated in the lungs may be the result of a purely environmental abnormality, such as the low partial pressure of oxygen in the atmosphere at high altitudes The commonest clinical causes of failure of oxygenation of blood in the lungs are mechanical, involving disease of the respiratory tract or paralysis of the muscles of respiration *Cyanosis*, a blue color of the skin, is a clinical sign which is often the first indication of hypoxia, of either pulmonary or circulatory origin It appears when the hemoglobin of the blood in the skin capillaries contains approximately 5 grams per 100 ml in the unoxygenated form Normally the content of unoxygenated hemoglobin in the capillary blood is about 2 5 grams per 100 ml The critical level of unoxygenated hemoglobin required to produce cyanosis is variable on account of differences in thickness, pigmentation, and vascularity of the skin Of greatest chemical interest are those causes of hypoxia which involve alteration of the hemoglobin molecule A number of noxious substances combine with or otherwise modify hemoglobin, depriving it of its property of reversible union with oxygen Some of these modifications of hemoglobin are more effective in producing cyanosis than is reduced hemoglobin itself

Methemoglobin, or ferrihemoglobin, is formed when the iron of the heme radical of hemoglobin is oxidized to the ferric (Fe^{+++}) state The conversion of hemoglobin to methemoglobin is reversible and does not in itself result in any damage to the red cell Methemoglobin, however, can not carry out the function of oxygen transport Small amounts of methemo- [illegible] detected [illegible] ethemo- [illegible] of any one of a rather considerable list of organic compounds (8) A very limited number of cases of congenital methemoglobinemia have been reported (8).

The presence of 1 5 grams of methemoglobin per 100 ml of blood is sufficient to produce cyanosis Methemoglobin may be detected qualitatively by a dark band in its absorption spectrum at 630 millimicrons This band can be caused to disappear by the addition of a little dilute cyanide solution Quantitative estimation of methemoglobin can be done simply by the use of the photoelectric colorimeter (7)

Hemoglobin which has been removed from the red cells and maintained in water solution exposed to the air undergoes a slow conversion to methemoglobin Hemoglobin which has been liberated from red cells into the plasma is partly converted to methemoglobin In plasma equilibrium is reached at about 50 per cent conversion In the red cell, however under normal conditions hemoglobin remains almost entirely in its normal condition with the iron in the ferrous state There is evidence (15) that the reduction of methemoglobin to normal hemoglobin is mediated in the red cell by an enzyme system which involves a flavin enzyme *methemoglobin reductase* and apparently both DPN and TPN Reduction of methemoglobin is accompanied by oxidation of glucose or lactate Alternative substrates shown to be effective in dog erythrocytes are fructose mannose galactose fumarate and malate A comparatively low content of the flavin enzyme has been observed in the red cells of patients with congenital methemoglobinemia

A very considerable degree of cyanosis produced by methemoglobinemia can be tolerated without the production of incapacitating symptoms The fatal concentration in man is not known Finch (8) has reported up to 50 per cent conversion to methemoglobin without the production of severe symptoms He recommends the slow intravenous injection of one mgm of methylene blue per kgm body weight in one per cent solution as the most satisfactory method for the treatment of severe methemoglobinemia Its action is catalytic to the normal enzymatic mechanism of the erythrocytes Congenital methemoglobinemia requires daily treatment with either methylene blue by mouth in dosages up to 300 mgm daily or ascorbic acid in dosages up to 500 mgm

Methemoglobin combines with the cyanide ion producing *cyanmethemoglobin* Dosages of cyanides otherwise lethal may be combated by the intravenous injection of sodium nitrite in dosages of 0 5 grams This results in the formation of methemoglobin which will combine with some of the cyanide and prevent its more dangerous combination with cytochrome oxidase The injection of nitrites should be followed by the injection of sodium thiosulfate in 25 gram dosage which will combine with the slowly liberated cyanide ion to form thiocyanate which is not dangerously toxic and which is readily excreted If sodium nitrite in a preparation suitable for intravenous administration is not available inhalation of amyl nitrite

is reported to be a satisfactory substitute. It should be recalled that this drug has a strong depressor action and may in itself cause unconsciousness.

Sulfhemoglobin is even more effective than methemoglobin in the production of cyanosis. The presence of 0.5 grams of sulfhemoglobin per 100 ml. of blood will produce a detectable bluish color in the patient's skin. Sulfhemoglobin is formed by the reaction of hydrogen sulfide with oxyhemoglobin. The chemical change in the hemoglobin molecule which is thus brought about is not clearly understood. Sulfhemoglobin can be identified by its absorption band at 618 millimicrons which does not disappear by the addition of cyanide. The use of cyanide differentiates this band from that of methemoglobin which is located very close to it. The formation of sulfhemoglobin, unlike that of methemoglobin, appears to be irreversible. Clinically, sulfhemoglobin is observed usually only in small and relatively insignificant amounts. It is most characteristic of so-called *enterogenous cyanosis*, which results from formation of abnormal amounts of hydrogen sulfide by bacterial action in the intestine. Enterogenous cyanosis can be produced easily in rabbits by the feeding of powdered sulfur (1), with demonstrable production of sulfhemoglobin.

Carbon monoxide hemoglobin resembles oxyhemoglobin very closely in its absorption spectrum, and in the type of union between the gas and the hemoglobin molecule. Like the reaction of hemoglobin with oxygen, the reaction of hemoglobin with carbon monoxide is reversible, but the equilibrium is much more in favor of the formation of carbon monoxide hemoglobin. At very low concentrations of carbon monoxide in the inspired air carbon monoxide hemoglobin tends to increase in the blood. The rate of increase depends upon the concentration of carbon monoxide in the air and upon the rate of pulmonary ventilation. Approximate equations have been established experimentally (9) which indicate the expected saturation of the blood after an exposure of t minutes: with the subject at rest, the per cent saturation of the blood with carbon monoxide will be $3t$ (per cent CO in inspired air); for light activity (pulse 80, ventilation 9.5 liters per minute), the coefficient becomes 5; for light work (pulse 110, ventilation 18 liters per minute), the coefficient is 8; and for heavy work (pulse 135, ventilation 30 liters per minute), the coefficient is 11. These generalizations apply when the concentration of CO in the inspired air is 0.02 per cent or more. At 0.01 per cent CO in the inspired air, the blood reaches a limit of saturation at about 7 per cent. By saturation with CO is meant the per cent of the total hemoglobin combined as carbon monoxide hemoglobin with proportional loss of oxygen capacity of the blood. The effect upon oxygen transport is actually more than proportional since the presence of carbon monoxide hemoglobin alters the dissociation curve of the remaining oxyhemoglobin, shifting the curve to the left and making it less sigmoid

This makes the remaining oxyhemoglobin less effective in unloading oxygen in the tissues A 50 per cent loss of hemoglobin by chronic hemorrhage may not in itself be incapacitating but 50 per cent saturation of hemoglobin with CO is close to the point of unconsciousness from hypoxia The presence of methemoglobin has an effect upon the dissociation curve of the remaining oxyhemoglobin qualitatively similar but quantitatively less than that of carbon monoxide hemoglobin If both substances are present the effects are additive (5)

Oxygen Toxicity

During the later years of the 19th century, Paul Bert and Lorrain Smith presented evidence that oxygen at partial pressures considerably above the normal had a toxic effect In Paul Bert's experiments a convulsive state was produced in his experimental birds Lorrain Smith demonstrated pulmonary irritation when experimental animals breathed oxygen at moderately high partial pressures over prolonged periods This work was largely neglected until about 1910 when human experimentation re-emphasized the fact that oxygen can be toxic The detailed effects of high partial pressures of oxygen are given in a review by Donald (6) In brief exposure to oxygen at partial pressure of 450 mm Hg will produce toxic effects The time required is shorter the higher the partial pressure of oxygen and is extremely variable among different human subjects The toxic effects appear to arise as the result of two separate mechanisms (a) depression of the mechanisms of carbon dioxide transport in the blood resulting in increased blood carbonic acid with production of a respiratory acidosis and (b) the oxidative destruction of certain important metabolic enzymes such as D amino acid oxidase xanthine oxidase, and particularly, pyruvate oxidase The respiration of brain tissue is irreversibly checked by high oxygen partial pressures The brain appears to be the most sensitive of all the tissues of the body to oxygen toxicity with the result that common symptoms of acute oxygen toxicity are of the nature of neurological manifestations Twitching particularly of the lips is an early sign which with continued exposure becomes more generalized until a convulsive state is reached From experiments *in vitro* we learn that next to the brain the spinal cord is most sensitive followed in order by liver testis kidney lung and muscle As a result of our knowledge of possibilities and dangers of the toxicity of oxygen at high pressures it is possible to control the partial pressures of oxygen during diving operations in such a manner as to minimize such dangers

CARBON DIOXIDE TRANSPORT BY THE BLOOD

Carbon dioxide originates in the tissues by the decarboxylation of organic acid metabolites This process is illustrated by the tricarboxylic acid

cycle The CO_2 diffuses freely in solution from its intracellular site of formation into interstitial fluid and blood plasma In these fluids, the reaction



$$H_2O + CO_2 \rightleftharpoons H_2CO_3$$

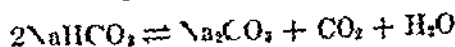
proceeds slowly towards equilibrium The velocity of this reaction—the hydration of carbon dioxide—when uncatalyzed is so slow that only an *insignificant amount of carbonic acid is formed from CO_2 in the extracellular fluid* Within certain cells, including the red cells of the blood carbon dioxide is rapidly hydrated by the action of a specific zinc-containing enzyme, *carbonic anhydrase* This enzyme is present in red cells but *not in plasma or interstitial fluid Within red cells, therefore rapid hydration* of CO_2 to H_2CO_3 or dehydration of H_2CO_3 to CO_2 can take place The direction of the reaction is determined by the partial pressure of CO

Carbon dioxide is taken up and released by the blood by no single simple mechanism As in the case of oxygen, *the over-all movement of CO_2 is* determined by differences of partial pressure which is highest in the tissues where CO_2 is produced and decreases as it passes through tissue fluids and blood and reaches the alveolar air For values of these partial pressures, see table 39 The partial pressure of CO_2 in blood is exerted only by that small fraction (2 to 2.5 ml per 100 ml blood) which is in solution as dissolved CO_2

When a sample of normal human arterial blood is acidified and placed in a Torricellian vacuum it will give up all of its free and combined CO_2 This amounts to 48.2 ml per 100 ml of blood with a standard deviation of 1.4 ml, measured under standard conditions of temperature and pressure (11) Venous blood taken from a resting subject has a total CO_2 content of 54.8 ml per 100 ml of blood with a standard deviation of 1.6 ml The arterio-venous difference of approximately 6.6 ml of CO_2 represents the amount eliminated from 100 ml of blood during its passage through the lungs and the amount picked up by the same amount of blood during its circuit through the tissues When the subject exercises, the arterio-venous difference is greatly increased

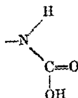
To convert *ml of CO_2 per 100 ml to millimols CO_2 per liter*, divide by 2.224, a figure based upon the fact that since CO_2 is not a perfect gas one mol of CO_2 occupies 22.24 liters under standard conditions of temperature and pressure The average total CO_2 content of arterial blood is therefore *21.7 millimols per liter and that of venous blood is 24.6 millimols per* liter In both venous and arterial blood, CO_2 can be identified in four forms physically dissolved CO_2, undissociated H_2CO_3, bicarbonate ion, HCO_3^- and as the carbamino group (formula I) formed by direct addition of CO_2 to amino groups of proteins

in the blood and the manner of its uptake by blood from tissues and release in the lungs is made more comprehensible by a brief review of behavior of simple solutions of this gas its hydrated form—carbonic a and the salts of carbonic acid Since the greatest part of the CO_2 of blood is in the form of bicarbonate ion we are particularly intereste this substance Solutions of sodium bicarbonate give up CO_2 slowly fo ing carbonate at room temperature



$$2NaHCO_3 \rightleftharpoons Na_2CO_3 + CO_2 + H_2O$$

This fact is worth keeping in mind for other reasons than to spin the of respiratory physiology Sodium bicarbonate is sometimes used in in venous medication—solutions of sodium bicarbonate can not be steril by boiling or autoclaving without the above reaction being greatly acc ated This results in dangerously increased alkalinity Furthermore fluids used in contact lenses are often buffered with bicarbonate Su



I Carbamino group

standing at room temperature causes an increase in alkalinity suffi to make the fluids irritating Note from the equation that the reacti limited to loss of half the CO_2 the reaction terminating with the version of bicarbonate to carbonate

When plasma is subjected to reduced pressure it will lose more CO_2 than will a simple bicarbonate solution of the same bicarbonate centration but will not lose it all There are acidic components in the pl which will to a degree liberate CO_2 from carbonate Whole blood jected to a Torricellian vacuum will lose all of its CO_2 the diffe between the behavior of plasma and whole blood in this respect i result of the presence of the acidic protein hemoglobin

Oxygenated hemoglobin is more dissociated as an acid—in other w it is a stronger acid—than unoxygenated hemoglobin One might the expect that there would be an increased hydrogen ion concentrati decreased pH in the blood as it becomes oxygenated in the lungs T not observed Instead the hydrogen ions react with bicarbonate to lit carbonic acid which breaks down accelerated by carbonic anhy into CO_2 and water The CO_2 diffuses into the alveolar air The same c in the opposite direction occurs in the tissues As oxyhemoglobin lo

oxygen, as a result of lower partial pressure of oxygen in the tissues, it becomes a weaker acid and therefore its hydrogen competes less effectively with the alkali metal cations of the blood for the bicarbonate radical. Hence in venous blood the concentration of alkali bicarbonates can be, and is, higher than in arterial blood. This sequence of events involving the increased acidity of hemoglobin when oxygenated is known as the *isohydric change* of hemoglobin, to emphasize the fact that there is no actual change in the pH of the blood.

In addition to the aid given to CO_2 transport by the isohydric change, hemoglobin also carries CO_2 in a more direct fashion. Approximately one fifth of the CO_2 of the blood travels in the form of *carbamino compounds*, formed by the direct union of carbon dioxide (not carbonic acid) with the amino groups of the blood proteins. Among the blood proteins hemoglobin



$$\text{Protein NH}_2 + CO_2 \rightleftharpoons \text{Protein NHCOOH}$$

is most significant in carbamino group formation, both on account of its greater amount in the blood than any other protein, and on account of its greater number of available amino groups. Oxygenation of hemoglobin causes liberation of a portion of the carbamino bound CO_2. Carbamino compounds were suspected when it was found that all the CO_2 could not be precipitated as barium carbonate. Carbon dioxide will combine in this fashion with simple amino compounds and with the negative ions of amino acids. It does not so combine with ammonium ions or the isoelectric ions of amino acids. Note that the carbamino reaction involves CO_2 directly. Carbonic acid or bicarbonate ion will not form carbamino compounds. The carbamino reaction is rapid and is not known to be catalyzed by any enzyme.

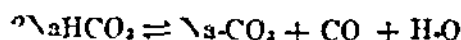
A third function of hemoglobin in carbon dioxide transport involves its position in the red cell. CO_2 can diffuse into red cells, and is there quickly hydrated enzymatically forming H_2CO_3, which is converted to bicarbonate by reaction with hemoglobin salts. The K^+ can not quickly leave the red



$$H_2CO_3 + K^+ + Hb^- \rightleftharpoons K^+ + HCO_3^- + HHb$$

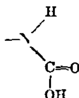
cell, but the HCO_3^- ions can and do, and an equal number of Cl^- or other anions enter by diffusion. This exchange occurs approaching as a limit the theoretical distribution calculated from the Gibbs Donnan theory of membrane equilibria. The net result of this *chloride shift* is increased alkalinity of the plasma which increases its ability to carry CO_2 as bicarbonate. The alkalinity of the plasma is increased since it loses a strong acid ion Cl^-, and gains an equivalent of a weak acid ion HCO_3^-. Reversal of the chloride shift occurs in the lungs initiated by diffusion of CO_2 out of the red cells in response to lowered partial pressures in the plasma.

in the blood and the manner of its uptake by blood from tissues and its release in the lungs is made more comprehensible by a brief review of the behavior of simple solutions of this gas, its hydrated form—carbonic acid and the salts of carbonic acid. Since the greatest part of the CO_2 of the blood is in the form of bicarbonate ion, we are particularly interested in this substance. Solutions of sodium bicarbonate give up CO_2 slowly forming carbonate at room temperature



$$2NaHCO_3 \rightleftharpoons Na_2CO_3 + CO_2 + H_2O$$

This fact is worth keeping in mind for other reasons than to explain theories of respiratory physiology. Sodium bicarbonate is sometimes used in intravenous medication—solutions of sodium bicarbonate can not be sterilized by boiling or autoclaving without the above reaction being greatly accelerated. This results in dangerously increased alkalinity. Furthermore the fluids used in contact lenses are often buffered with bicarbonate. Simple



I Carbamino group

standing at room temperature causes an increase in alkalinity sufficient to make the fluids irritating. Note from the equation that the reaction is limited to loss of half the CO_2, the reaction terminating with the conversion of bicarbonate to carbonate.

When plasma is subjected to reduced pressure, it will lose more of its CO_2 than will a simple bicarbonate solution of the same bicarbonate concentration but will not lose it all. There are acidic components in the plasma which will to a degree liberate CO_2 from carbonate. Whole blood subjected to a Torricellian vacuum will lose all of its CO_2; the difference between the behavior of plasma and whole blood in this respect is the result of the presence of the acidic protein hemoglobin.

Oxygenated hemoglobin is more dissociated as an acid—in other words it is a stronger acid—than unoxygenated hemoglobin. One might therefore expect that there would be an increased hydrogen ion concentration or decreased pH in the blood as it becomes oxygenated in the lungs. This is not observed. Instead, the hydrogen ions react with bicarbonate to liberate carbonic acid, which breaks down, accelerated by carbonic anhydrase, into CO_2 and water. The CO_2 diffuses into the alveolar air. The same change in the opposite direction occurs in the tissues. As oxyhemoglobin loses

oxygen as a result of lower partial pressure of oxygen in the tissues, it becomes a weaker acid and therefore its hydrogen competes less effectively with the alkali metal cations of the blood for the bicarbonate radical. Hence in venous blood the concentration of alkali bicarbonates can be, and is, higher than in arterial blood. This sequence of events involving the increased acidity of hemoglobin when oxygenated is known as the *isohydric change* of hemoglobin to emphasize the fact that there is no actual change in the pH of the blood.

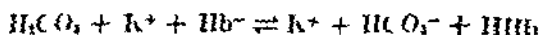
In addition to the aid given to CO_2 transport by the isohydric change, hemoglobin also carries CO_2 in a more direct fashion. Approximately one fifth of the CO_2 of the blood travels in the form of *carbamino compounds*, formed by the direct union of carbon dioxide (not carbonic acid) with the *amino groups of the blood proteins. Among the blood proteins hemoglobin*



$$\text{Protein NH}_2 + CO_2 \rightleftharpoons \text{Protein NHCOOH}$$

is most significant in carbamino group formation, both on account of its greater amount in the blood than any other protein, and on account of its greater number of available amino groups. Oxygenation of hemoglobin causes liberation of a portion of the carbamino bound CO_2. Carbamino compounds were suspected when it was found that all the CO_2 could not be precipitated as barium carbonate. Carbon dioxide will combine in this fashion with simple amino compounds and with the negative ions of amino acids. It does not so combine with ammonium ions or the isoelectric ions of amino acids. Note that the carbamino reaction involves CO_2 directly. Carbonic acid or bicarbonate ion will not form carbamino compounds. *The carbamino reaction is rapid and is not known to be catalyzed by any enzyme.*

A third function of hemoglobin in carbon dioxide transport involves its position in the red cell. CO_2 can diffuse into red cells and is there quickly hydrated enzymatically, forming H_2CO_3, which is converted to bicarbonate by reaction with hemoglobin salts. The K^+ can not quickly leave the red



$$H_2CO_3 + K^+ + Hb^- \rightleftharpoons K^+ + HCO_3^- + HHb$$

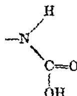
cell but the HCO_3^- ions can and do, and an equal number of Cl^- or other anions enter by diffusion. This exchange occurs, approaching as a limit the theoretical distribution calculated from the Gibbs-Donnan theory of membrane equilibria. The net result of this *chloride shift* is increased alkalinity of the plasma, which increases its ability to carry CO_2 as bicarbonate. *The alkalinity of the plasma is increased since it loses a strong acid ion,* Cl^-, and gains an equivalent of a weak acid ion, HCO_3^-. Reversal of the chloride shift occurs in the lungs, initiated by diffusion of CO_2 out of the red cells in response to lowered partial pressures in the plasma.

in the blood and the manner of its uptake by blood from tissues and its release in the lungs is made more comprehensible by a brief review of the behavior of simple solutions of this gas, its hydrated form—carbonic acid and the salts of carbonic acid Since the greatest part of the CO_2 of the blood is in the form of bicarbonate ion, we are particularly interested in this substance Solutions of sodium bicarbonate give up CO_2 slowly, form rbonate, at room temperature



$$2NaHCO_3 \rightleftharpoons Na_2CO_3 + CO_2 + H_2O$$

fact is worth keeping in mind for other reasons than to spin theories of respiratory physiology Sodium bicarbonate is sometimes used in intravenous medication—*solutions of sodium bicarbonate can not be sterilized* by boiling or autoclaving without the above reaction being greatly accelerated This results in dangerously increased alkalinity Furthermore, the fluids used in contact lenses are often buffered with bicarbonate Simple



I Carbamino group

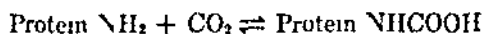
standing at room temperature causes an increase in alkalinity sufficient to make the fluids irritating Note from the equation that the reaction is limited to loss of half the CO_2, the reaction terminating with the conversion of bicarbonate to carbonate

When plasma is subjected to reduced pressure, it will lose more of its CO_2 than will a simple bicarbonate solution of the same bicarbonate concentration, but will not lose it all There are acidic components in the plasma which will to a degree liberate CO_2 from carbonate Whole blood subjected to a Torricellian vacuum, will lose all of its CO_2, the difference between the behavior of plasma and whole blood in this respect is the result of the presence of the acidic protein hemoglobin

Oxygenated hemoglobin is more dissociated as an acid—in other words it is a stronger acid—than unoxygenated hemoglobin One might therefore expect that there would be an increased hydrogen ion concentration or decreased pH in the blood as it becomes oxygenated in the lungs This is not observed Instead, the hydrogen ions react with bicarbonate to liberate carbonic acid, which breaks down, accelerated by carbonic anhydrase into CO_2 and water The CO_2 diffuses into the alveolar air The same change in the opposite direction occurs in the tissues As oxyhemoglobin loses its

oxygen as a result of lower partial pressure of oxygen in the tissues, it becomes a weaker acid and therefore its hydrogen competes less effectively with the alkali metal cations of the blood for the bicarbonate radical. Hence in venous blood the concentration of alkali bicarbonates can be and is, higher than in arterial blood. This sequence of events involving the increased acidity of hemoglobin when oxygenated is known as the *isohydric change* of hemoglobin, to emphasize the fact that there is no actual change in the pH of the blood.

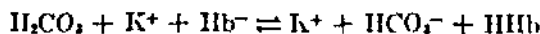
In addition to the aid given to CO_2 transport by the isohydric change, hemoglobin also carries CO_2 in a more direct fashion. Approximately one fifth of the CO_2 of the blood travels in the form of *carbamino compounds*, formed by the direct union of carbon dioxide (not carbonic acid) with the amino groups of the blood proteins. Among the blood proteins, hemoglobin



$$\text{Protein}\ NH_2 + CO_2 \rightleftharpoons \text{Protein}\ NHCOOH$$

is most significant in carbamino group formation, both on account of its greater amount in the blood than any other protein, and on account of its greater number of available amino group. Oxygenation of hemoglobin causes liberation of a portion of the carbamino bound CO_2. Carbamino compounds were suspected when it was found that all the CO_2 could not be precipitated as barium carbonate. Carbon dioxide will combine in this fashion with simple amino compounds and with the negative ions of amino acids. It does not so combine with ammonium ions or the isoelectric ions of amino acids. Note that the carbamino reaction involves CO_2 directly. Carbonic acid or bicarbonate ion will not form carbamino compounds. The carbamino reaction is rapid and is not known to be catalyzed by any enzyme.

A third function of hemoglobin in carbon dioxide transport involves its position in the red cell. CO_2 can diffuse into red cells, and is there quickly hydrated enzymatically, forming H_2CO_3 which is converted to bicarbonate by reaction with hemoglobin salts. The K^+ can not quickly leave the red



$$H_2CO_3 + K^+ + Hb^- \rightleftharpoons K^+ + HCO_3^- + HHb$$

cell but the HCO_3^- ions can and do, and an equal number of Cl^- or other anions enter by diffusion. This exchange occurs approaching as a limit the theoretical distribution calculated from the Gibbs Donnan theory of membrane equilibria. The net result of this *chloride shift* is increased alkalinity of the plasma which increases its ability to carry CO_2 as bicarbonate. The alkalinity of the plasma is increased since it loses a strong acid ion, Cl^-, and gains an equivalent of a weak acid ion HCO_3^-. Reversal of the chloride shift occurs in the lungs initiated by diffusion of CO_2 out of the red cells in response to lowered partial pressures in the plasma.

THE MAINTENANCE OF NEUTRALITY

Between the approximate limits of pH 6 8 and pH 7 8 it is possible for human cells to remain alive The much narrower limits of pH 7 32 and pH 7 46 mark the extremes of variation observed in the blood of the healthy resting human subject (11) This remarkable constancy of pH is the result of the co-ordinated action of four mechanisms (a) the buffers of the cells and the extracellular fluids (b) the elimination by the lungs of CO_2 which is the anhydride of an acid H_2CO_3 (c) the elimination in the urine of both acids and bases, and (d) the formation by the kidney of ammonia from glutamine and from amino acids in response to the stimulus of increased acidity

Buffer systems are composed of either a weak acid or a weak base in the same aqueous solution with the salt of that weak acid or base The addition of acid or base to such a system causes a much smaller change in the pH of the system compared to the change brought about by the addition of the same amount of acid or base to plain water or to NaCl solution The pH of such a system is stated by the Henderson Hasselbalch equation

$$pH = pK + \log \frac{(\text{salt})}{(\text{acid})}$$

It will be recalled that pH is the logarithm of the reciprocal of the hydrogen ion concentration Similarly pK is the logarithm of the reciprocal of the ionization constant of the weak acid It is also equal to the pH of the buffer solution when the salt and the acid are present in equivalent amounts In this case the fraction of the concentrations becomes equal to one its logarithm becomes equal to zero and pH equals pK One would judge from this equation that since pH is determined for a system containing a given acid and its salt solely by the ratio of the salt to the acid that changes in concentration of the salt and acid while the ratio is still maintained would have no effect upon the pH Dilution of a buffer system therefore *should not change its pH This is true within limits* The ionization constants of most acids however, are not actually constant over wide ranges of concentration Therefore one should be careful to pick a value of K consistent with the concentration used Although this equation is very satisfactory in predicting the behavior of most buffer systems there are other errors which prevent its exact application in all circumstances Anyone planning to do precise work involving buffer systems should first consult a specialized monograph on the subject such as that of Clark (3)

It is obvious that no buffer system can withstand the addition of an indefinite amount of acid or base without change of pH The addition of strong acid converts salt to weak acid the addition of strong base converts weak acid to salt Each such addition alters the ratio and alters the pH

The buffering effect is dependent upon the fact that the addition of strong acid produces an effect equal to the addition of an equivalent amount of weak acid and that the addition of strong base produces a result equivalent to that of the removal or neutralization of an equivalent amount of weak acid The buffer capacity or ability to handle additions of acid or base without marked change in pH varies directly with the concentrations of the original components of the buffer system

The principal buffer system of both blood and tissues is composed of protein acting as a weak acid in the presence of salts of protein Other significant buffer systems are composed of carbonic acid in the presence of bicarbonate, and dihydrogen phosphate in the presence of monohydrogen phosphate

Pulmonary elimination of CO_2 adds enormously to the efficiency of the carbonic acid bicarbonate buffer Setting up the Henderson Hasselbalch equation for the conditions prevailing in the blood, it can be seen that this

$$7\,4 = 6\,1 + \log \frac{20}{1}$$

buffer system is operating at a considerable distance from its midpoint, or pK value which is the point of maximum buffering efficiency Nevertheless, the fact that when acid enters the plasma from metabolic or other sources the H_2CO_3 liberated is in part converted to CO_2 and eliminated by the lungs, permits this buffer system to continue acting with only minimal changes of pH The *renal excretion of acids and bases* and the *renal formation of ammonia* will be discussed in Chapter 18 It should be pointed out here that the renal mechanisms are much slower in their response than is the mechanism of excretion of excess CO_2 by the lungs

One of the above mechanisms, ammonia formation, is effective against excess acidity only The others are involved in the defense against both acidity and alkalinity The passive elimination of CO_2 with increased acidity of the blood is augmented by the action of the respiratory center The rate and amplitude of pulmonary ventilation are closely regulated towards maintaining a constant partial pressure of CO_2 through the respiratory center in the medulla Increases in partial pressure of CO_2 and increases in acidity of the blood are directly stimulatory to the respiratory center, and normally lead to increased rate and amplitude of respiratory movements Decreases in blood CO_2 and acidity have an opposite effect, diminishing pulmonary ventilation In the carotid and aortic bodies are located chemoreceptors which are stimulated most effectively by decreased partial pressure of O_2 in the blood, and which in turn stimulate the respiratory center The carotid and aortic receptors are less sensitive under normal circumstances, than the respiratory center itself

Acid products are in excess of basic products in the metabolic scheme Foodstuffs do not contain appreciable amounts of metallic hydroxides, basic phosphates or preformed carbonates or bicarbonates Salts of organic acids occur in abundance Many of the organic acid radicals are metabolizable such as acetate malate citrate, and succinate When these are converted to CO_2 and water by physiological oxidative processes the metallic cation remains paired with the bicarbonate ion formed by the hydration of CO_2 The hydrolysis of the alkali and alkaline-earth bicarbonates and of similar salts of other weak acids is the only significant source of hydroxyl ion in cells or body fluids Acids, contrariwise, are produced in abundance The daily output of CO_2 is the equivalent if all hydrated to H_2CO_3 (which it is not) of 20 to 40 liters of normal acid Sulfuric and phosphoric acids are produced by the oxidation of the S of the diet chiefly proteins, and the P of the diet phospholipids and phosphoproteins The majority of the intermediates of carbohydrate and lipid metabolism are organic acids the carboxyl groups of which ultimately form CO_2 Other acid radicals include Cl^- which can affect acid base balance by its unequal distribution between cells and extracellular fluid and protein, which at the pH of the internal environment acts as a negative ion The preponderance of acids among the metabolic products of the body demands a dependable supply of "base", meaning alkali or alkaline earth metal cations to maintain neutrality The term "base" is customarily used in this sense by physiologists and physiological chemists

Alkaline reserve in the broad meaning of the term indicates the total "base" of the body—all the intracellular and extracellular alkali or alkaline earth metal cations present and paired with acid radicals This quantity can not be measured in the intact body It is paralleled by another quantity which can be measured the CO_2 capacity of the plasma This measurement gives the quantity of CO_2 present in the plasma when the plasma is saturated with CO_2 at the oxygen and CO_2 partial pressures of normal alveolar air While all the CO_2 of the plasma is not present as bicarbonate ion the fraction so combined is the largest and most variable fraction, and is directly proportional to the total "base" of the plasma The other fractions dissolved CO_2 and free H_2CO_3, are smaller and relatively constant Hence the total CO_2 capacity varies with the amount of "base" present The CO_2 capacity is measured by first equilibrating plasma with either actual alveolar air from the lungs of the analyst or a mixture of gases with the composition of alveolar air A measured sample of the equilibrated plasma is then introduced into a mercury filled tube where it is mixed with acid and subjected to a Torricellian vacuum The extracted CO_2 is either subjected to atmospheric pressure and the volume measured, or else in a slightly different instrument is brought to a definite volume and its pressure

measured The CO_2 capacity may be calculated either as volumes per cent of CO_2 (see page 615) or as milliequivalents HCO_3^- per liter plasma. The latter terminology is more common in current use The normal average value is 60 volumes per cent of which 95 per cent is actually from bicarbonate ion the remainder being dissolved CO_2 or free H_2CO_3 Expressed in milliequivalents of HCO_3 per liter (or identically in millimols of CO_2 per liter) the corresponding value is 27 The CO_2 *combining power* of plasma is the CO_2 capacity from which has been subtracted a correction of 1 2 mEq per liter This correction represents the concentration of undissociated H_2CO_3 in plasma at a partial pressure of CO_2 of 40 mm Hg The corrected value represents the bicarbonate content or "alkaline reserve". Total CO_2 *content* of the true plasma of blood as it is drawn from the subject is normally 24 to 33 mEq per liter with an average value of 28 2 mEq (14) True plasma is plasma separated from the cells of the blood under conditions of strict exclusion of air so that no loss of CO_2 can take place The CO_2 content of true plasma is the single most useful measurement in the evaluation of clinical states of acid base imbalance Actually, no single analytical determination will give a fully reliable assessment of such clinical states (14) If significant changes in the mineral ions of the blood have not taken place measurement of the CO_2 content and of pH will establish the nature of the disturbance Since Na^+ is the most important cation of the plasma as far as acid base balance is concerned its estimation (by flame photometry or by precipitation with uranyl zinc acetate) completes the evaluation

STATES OF ACID-BASE IMBALANCE

Some confusion still exists in the definition of the terms used in describing abnormalities or failures of neutrality regulation Previous definitions based upon variations in alkaline reserve as measured by CO_2 combining power may well be abandoned along with that technique, which offers no information not yielded by measurement of CO_2 content along with pH Na^+ concentration or both We can best define *acidosis* as an excess of acid or deficiency of base (meaning mineral cations) which, if not compensated by the body's regulatory mechanisms results in *a decreased pH* A fall in pH to a level of about 7 results in a state of coma, followed by functional failure of the heart and the respiratory centers Hyperpnea and tachycardia may be significant earlier symptoms *Respiratory acidosis* results from failure of pulmonary elimination of CO_2 and is manifested by increased concentrations of CO_2 and of HCO_3 in tissues and body fluids The CO_2 content is always increased above normal, but the CO_2 capacity or CO_2 combining power (alkaline reserve) may remain normal or may be moderately increased It is possible in respiratory acidosis for the CO

Acid products are in excess of basic products in the metabolic scheme Foodstuffs do not contain appreciable amounts of metallic hydroxides, basic phosphates, or preformed carbonates or bicarbonates Salts of organic acids occur in abundance Many of the organic acid radicals are metabolizable, such as acetate, malate, citrate, and succinate When these are converted to CO_2 and water by physiological oxidative processes, the metallic cation remains paired with the bicarbonate ion formed by the hydration of CO_2 The hydrolysis of the alkali and alkaline earth bicarbonates and of similar salts of other weak acids is the only significant source of *hydroxyl ion in cells* or body fluids Acids, contrariwise, are produced in abundance* *The daily output of CO_2* is the equivalent, if all hydrated to H_2CO_3 (which it is not), of 20 to 40 liters of normal acid Sulfuric and phosphoric acids are produced by the oxidation of the S of the diet, chiefly proteins, and the P of the diet, phospholipids and phosphoproteins The majority of the intermediates of carbohydrate and lipid metabolism are organic acids, the carboxyl groups of which ultimately form CO_2 Other acid radicals include Cl^-, which can affect acid base balance by its unequal distribution between cells and extracellular fluid, and protein, which at the pH of the internal environment acts as a negative ion The preponderance of acids among the metabolic products of the body demands a dependable supply of "base", meaning alkali or alkaline earth metal cations, to maintain neutrality The term "base" is customarily used in this sense by physiologists and physiological chemists

Alkaline reserve in the broad meaning of the term indicates the total "base" of the body—all the intracellular and extracellular alkali or alkaline earth metal cations present and paired with acid radicals This quantity can not be measured in the intact body It is paralleled by another quantity which can be measured, the CO_2 *capacity* of the plasma This measurement gives the quantity of CO_2 present in the plasma when the plasma is saturated with CO_2 at the oxygen and CO_2 partial pressures of normal alveolar air While all the CO_2 of the plasma is not present as bicarbonate ion, the fraction so combined is the largest and most variable fraction, and is directly proportional to the total "base" of the plasma The other fractions dissolved CO_2 and free H_2CO_3, are smaller and relatively constant Hence the total CO_2 capacity varies with the amount of "base" present The CO_2 capacity is measured by first equilibrating plasma with either actual alveolar air from the lungs of the analyst or a mixture of gases with the composition of alveolar air A measured sample of the equilibrated plasma is then introduced into a mercury filled tube where it is mixed with acid and subjected to a Torricellian vacuum The extracted CO_2 is either subjected to atmospheric pressure and the volume measured, or else in a slightly different instrument is brought to a definite volume and its pressure

measured The CO_2 capacity may be calculated either as volumes per cent of CO_2 (see page 615) or as milliequivalents HCO_3^- per liter plasma. The latter terminology is more common in current use The normal average value is 60 volumes per cent, of which 95 per cent is actually from bicarbonate ion, the remainder being dissolved CO_2 or free H_2CO_3 Expressed in milliequivalents of HCO_3 per liter (or identically in millimols of CO_2 per liter), the corresponding value is 27 The CO_2 *combining power* of plasma is the CO_2 capacity from which has been subtracted a correction of 1 2 mEq per liter This correction represents the concentration of undissociated H_2CO_3 in plasma at a partial pressure of CO_2 of 40 mm Hg The corrected value represents the bicarbonate content or "alkaline reserve". *Total CO_2 content of the true plasma of blood as it is drawn from the subject* is normally 24 to 33 mEq per liter, with an average value of 28 2 mEq (14) True plasma is plasma separated from the cells of the blood under conditions of strict exclusion of air so that no loss of CO_2 can take place The CO_2 content of true plasma is the single most useful measurement in the evaluation of clinical states of acid base imbalance Actually, no single analytical determination will give a fully reliable assessment of such clinical states (14) If significant changes in the mineral ions of the blood have not taken place, measurement of the CO_2 content and of pH will establish the nature of the disturbance Since Na^+ is the most important cation of the plasma as far as acid base balance is concerned, its estimation (by flame photometry or by precipitation with uranyl zinc acetate) completes the evaluation

STATES OF ACID-BASE IMBALANCE

Some confusion still exists in the definition of the terms used in describing abnormalities or failures of neutrality regulation Previous definitions based upon variations in "alkaline reserve" as measured by CO_2 combining power may well be abandoned along with that technique, which offers no information not yielded by measurement of CO_2 content along with pH, Na^+ concentration, or both We can best define *acidosis* as an excess of acid or deficiency of "base" (meaning mineral cations) which, if not compensated by the body's regulatory mechanisms, results in *a decreased pH* A fall in pH to a level of about 7 results in a state of coma, followed by functional failure of the heart and the respiratory centers Hyperpnea and tachycardia may be significant earlier symptoms *Respiratory acidosis* results from failure of pulmonary elimination of CO_2 and is manifested by increased concentrations of CO_2 and of H_2CO_3 in tissues and body fluids The *CO_2 content is always increased above normal, but the CO_2 capacity* or CO_2 combining power ("alkaline reserve") may remain normal or may be moderately increased It is possible in respiratory acidosis for the CO_2

content of blood plasma to be greater than its CO_2 capacity This apparent impossibility is explained by the fact that CO_2 capacity *is arbitrarily* measured at a partial pressure of CO_2 equal to that in normal alveolar air With failure to eliminate CO the plasma is subjected to higher parti l pressures of that gas

Respiratory acidosis results from and accompanies serious disease of the respiratory organs such as pneumonia or respiratory paralysis in which the danger from hypoxia far outweighs the danger of acidosis The diffusibility of CO_2 is 25 times that of O_2 hence correction of hypoxia will usually automatically correct respiratory acidosis Patients with respiratory acidosis do not always respond well however to therapy with high concentrations of oxygen (2) Long continued increases of acidity or of CO_2 partial pressure in the arterial blood will *sometimes depress the respiratory* center so that it no longer responds to these changes which are stimulatory to pulmonary ventilation under usual circumstances In this situation pulmonary ventilation is maintained chiefly by the stimulus of hypoxia acting through the aortic and carotid chemoreceptors If oxygen is given to a patient in this state pulmonary ventilation diminishes and the partial pressure of CO_2 in the blood increases further aggravating the pre-existing respiratory acidosis *Metabolic acidosis* is a more urgent clinical problem Here there is either a loss of base resulting from excessive excretions or direct loss of body fluids, or el e a surplus of acid The CO_2 content is invariably below normal There is either an absolute or relative decrease of the true alkaline reserve and a decrease in the CO_2 capacity of the plasma since a portion of the base normally available for CO_2 transport is either lost or bound by excess of non volatile acid Causes of metabolic acidosis *other than loss of fluids* include ketosis (see Chapter 13) failure of excretion of acid waste products by diseased kidneys or *failure of renal* circulation and failure of gluconeogenesis from lactic acid *in terminal* stages of liver disease and in asphyxial states resulting in accumulation of lactic acid To the group of metabolic acidoses might well be added *therapeutic acidosis* where the mechanism is similar but the result of administration of acidifying drugs such as ammonium calcium or magnesium chlorides Ammonium chloride is acidifying since the ammonia portion is *promptly converted to urea* by the liver leaving a residue of HCl Calcium chloride has up to 75 per cent of the acidifying effect of an equivalent dosage of HCl since the Ca^{++} *is excreted chiefly* by the fecal route The same is true to a somewhat less degree of magnesium chloride The general therapeutic approach to situations involving metabolic acidosis includes removal of the cause and replacement of base and water

Alkalosis is the opposite of acidosis and may therefore be defined as a deficiency of acid or an excess of base (metallic cations) which unless

compensated brings about *an increase in pH* Alkalosis may be *respiratory* from overventilation which may occur in febrile or psychotic states or during inhalation anesthesia In respiratory alkalosis the CO_2 content of the blood plasma is diminished Alkalosis may be *metabolic* as from loss of gastric HCl by vomiting or *therapeutic* as from administration of soluble alkaline salts such as $NaHCO_3$ In metabolic or in therapeutic alkalosis the plasma CO_2 content is increased The most serious result of alkalosis of any type is muscular hyperirritability which at a plasma pH of about 7 6 becomes a state of tetany

REFERENCES

1 Barkan G and Walker B S Sulfhem gl bin formatic l labile iron in vitro and in vivo Sci 96 66–68 1942

2 Beale H D *et al* D lirium and com ipitated by ygen in bronchial asthma complicated by respiratory acid New Englan J Med 244 710–714 1951

3 Clark W M *The D t ation of Hyd Ions* 3rd edit Baltimore Md, The Williams & W s Co 1928

4 Comroe J H Jr Dripps R D Jr The oxygen tension of arterial blood and alveolar air in n d human subjects A n J Physiol 142 700–707 1944

5 Darling R C R hton F J W The effect of methemoglobin on the equilibrium between oxygen and hemogl Am J Physiol 137 56–68 1942

6 Donald K W Ox g n p sing in man Brit M J, 1947, I 667–672 712 717 1947

7 Evelyn K A Malloy H T Micr determination of oxyhemoglobin methemoglobin and sulfhemoglobin in a single sample of blood J Biol Chem 126 655–662 1938

8 Finch C A Methemoglobinemia and sulfhemoglobinemia New England J Med 239 470–478 1948

9 Forbes W H *et al* The rate of carbon m xide uptake by normal men Am J Physiol 143 594–608 1945

10 Gemmill C L The relationship betwee alveolar air oxygen tensions and arterial blood oxygen saturations in man uring work at altitude J Aviation Med 18 483–494 1947

11 Gibbs F I *et al* Arterial and cerebral ven us blood arterial venous differences in man J Biol Chem 144 325–332 1942

12 Lilienthal J I Jr *et al* An experim l analysis in man of the oxygen pressure gradient from alveolar air to arterial blood during rest and exercise at sea level and at ltitude Am J Physi l 147 199–216 1946

13 Pitts R F Acid b se regulation by the kidneys Am J Med, 9 356–372 1950

14 Singer R B and Hastings A B An improved clinical method for the estimation of disturbances in the acid base balance of human blood Medicine 27 223 212 1948

15 Spicer S S *et al* Studies *in vitro* on methemoglobin reduction in dog erythrocytes J Biol Chem 177 217 230 1949

CHAPTER 17

Heat and Work

It has long been known that the production of heat by friction shows that energy in the form of work can be converted into heat. The experiments of *Joule* and *later workers showed that no matter how the work* was applied, if heat loss was guarded against and unavoidable losses corrected for, exactly the same amount of heat always appeared in correspondence to the expenditure of a given amount of work. To give concrete examples, it was found that the work involved in lifting a one pound weight 778 feet was the equivalent of the heat required to increase the temperature of one pound of water one degree Fahrenheit. Or, in metric units, 427 kgm. meters was the equivalent of one Kcal. of heat. By showing that work and heat were interconvertible in a fixed ratio, these measurements served to prove the validity of the law of conservation of energy.

Previous to this the invention of the steam engine had already showed that it was possible to bring about the reverse transformation of heat into work. But systematic study of the problem revealed a very important difference. *The transformation of work into heat can be arranged so as to* give a 100 per cent yield, but the reverse is not true. *Steam engines never* convert more than a fraction of the heat of their fuel into work, *and the* French engineer Sadi Carnot showed that they never *could because of* inherent physical limitations to the process. Some *comprehension of the* second law of thermodynamics (see Appendix *III*) *and the concept of entropy is needed for a full understanding* of this proof, which we shall not attempt to give here. Let it suffice to say that Carnot showed that any *engine given an amount of heat* Q *and taking in its steam at a temperature* T_2 and discharging it at a lower temperature T_1, would never give an *amount of work* W *greater than*

$$W = \frac{Q(T_2 - T_1)}{T_2}$$

where the temperatures T_2 and T_1 are given in degrees absolute *and* Q *and* W are both expressed in identical units—say calories.

It can easily be seen that for actual engines *W* can *never be more than*

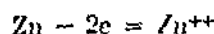
a fraction of Q (since T_1 is never zero) and in fact no saturated steam engine operating with a boiler pressure of for example 163 pounds per square inch can ever convert more than 18.5 per cent of the heat into work. *Even this is computed on the basis of the heat actually in the steam coming into the engine.* This is of course by no means all of the heat potentially available from the fuel. Some heat is lost by radiation and even more goes up the flue with the smoke. The efficiency of the boiler in transferring the heat of combustion to the engine may not be over 70 per cent. Thus the over-all maximum efficiency of the installation would be only 70 per cent of 18.5, or about 13 per cent. This is for a typical stationary steam plant. Steam locomotives probably do not utilize over 8 per cent of the energy available potentially in the fuel they burn. Steam engines with a very high temperature of operation (T_2) may show an efficiency as high as 24 per cent and one form of the internal combustion engine the diesel, may sometimes give efficiencies as high as 40 per cent.

It was natural for the early physiologists and biochemists seeing the triumphs of the new science of thermodynamics in the field of engineering, to ask themselves—How efficient is the human body as a converter into work of the heat available from the combustion of the foodstuffs? For it was apparent that all animals including man must eat in order to live and work, and must eat more when the work is heavy. An obvious way of answering this question was to determine the calorific value of the foodstuffs burned in the body during a given period of work and to compare this with the work produced, expressed in heat units. The answer obtained was that *the human body was more efficient mechanically than* a steam locomotive but certainly not 100 per cent efficient. This was not too surprising for it was known that the efficiency of the locomotive is low, and thermodynamics as we have seen shows that 100 per cent conversion of heat into work is impossible by any machine.

However although it was perfectly natural for the early workers in our field to ask themselves the above question and to attempt to solve it in the manner indicated it was nevertheless entirely the wrong question. The body has no power whatever of converting heat into work. A heat engine has for it makes use of some of the heat produced by burning its fuel to warm and thus expand a confined gas. The expansion of this confined gas does work, and because of the conversion of some of the energy contained in it into work, the gas ends up at a lower temperature (T_1) than at the start of the work cycle. The two temperatures of the gas are the T_2 and T_1 of the above equation. But the body operates in an entirely different way. It is not a heat engine and the laws of heat engines do not apply to it. *The more general laws of thermodynamics, originally based on* a study of heat engines do apply however and we can predict now that

even the body is never going to be able to convert 100 per cent of the energy potentially available in its foodstuffs into work It is easy to see why the body can not be a heat engine For one thing it keeps all its parts at approximately the same temperature, and thus the temperature gradient T_2 minus T_1 which is essential for the operation of the heat engine, is lacking

How does the body produce work? From previous chapters it was apparent that the body makes use of the energy of its foodstuffs by oxidizing them at constant temperature by a series of enzyme reactions The maximum possible work to be obtained from a chemical reaction depends upon the quantity called in thermodynamics the free energy of the reaction (See Appendix III) Some electrical cells can convert a very high percentage of the free energy of the chemical reaction that runs them into electrical energy Therefore, since the body is essentially an oxidation reduction cell producing work (instead of electrical energy, for the most part) directly, we might expect rather high mechanical efficiencies from it For instance we could compare the body to an electric battery coupled with an electric motor although the analogy is probably not perfect, since the body seems to convert the chemical energy of its foodstuffs directly into work (although producing a small amount of electricity also), whereas the electric cell produces primarily electric energy which is converted by the motor into work In an ordinary dry cell metallic zinc is consumed, and thus acts as the ' fuel ' of the cell, very much as the food stuffs of the body act as fuels In both cases the temperature remains nearly constant during the operation of the system and in both cases the mechanical efficiency can theoretically be quite high The essential reaction in a dry cell is



$$Zn - 2e = Zn^{++}$$

The metallic zinc is oxidized to its ions and electrons are transferred as a result Various complexities which need not concern us here make it difficult to compute the efficiency of this cell exactly, but the high efficiency of electric cells is indicated by the study of storage batteries such as those used in automobiles where one can compare directly the amount of electrical energy which went into charging the battery with the amount recovered on discharging it The chemical reactions vary with the type of cell used, but the efficiency of the ordinary lead storage battery, when discharged slowly, has been found to be as high as 95 per cent Obviously a high proportion of the free energy of the chemical reaction involved in the discharge of the cell is converted into work Undesirable secondary reactions keep the efficiency from being higher, and also limit the useful life of the cell, under ordinary conditions, to about two years

Electric motors may be constructed which convert 80 per cent or more of the electrical energy fed into them into mechanical work Thus such a motor, coupled to a good storage battery might yield a mechanical efficiency of 0 80 × 95 or 76 per cent One might possibly expect the human body to show similar high efficiencies

Various measurements have been made of the efficiency of the human body while it was performing work, and the results indicate that the body is mechanically more efficient than a steam locomotive (a result which surprised the early biochemists but does not now surprise us), but that it is not as efficient as a battery motor combination, or even the best internal combustion engines (diesels) A few words about the methods of computing the efficiency of the human body, and some explanation of the results, seem to be in order

For any machine we have the relation

$$\text{Input} = \text{Output} + \text{Work lost by friction} + \text{Other losses}$$

The efficiency is the ratio output input, usually expressed as a percentage

We can measure the work output of the human body by the weight lifted through a given height, or in other ways We can measure the input into the body if we know the calorific value of the various foodstuffs when oxidized in the body A maximum value is obtained by burning the foods directly with oxygen in the bomb calorimeter, and measuring the heat evolved The following results are obtained for the three classes of food stuffs

Class of foodstuff	*Kcal/gram dry weight*
Carbohydrate	4 10
Fat	9 45
Protein	5 65

Since the first two classes of foodstuffs are oxidized as completely in the body as in the bomb calorimeter, we can obtain the calorific values for human utilization merely by correcting for the imperfect absorption of the foods It has been estimated that on the average 98 per cent of the carbo hydrate is absorbed 95 per cent of the fat, and 92 per cent of the protein To obtain the calorific value of the proteins in the body we must allow also for the fact that they are not completely oxidized In a classic experiment whose results are still quoted, Rubner (3) found that about 75 per cent of the potential calorific value of proteins was realized in the animal body (see page 638) Making these corrections, and rounding off the results to whole numbers since the precise figures may vary somewhat with the particular food within any one of the classes, we obtain the following very important and easily memorized values for the calorific value of the three classes of food within the animal body

Class of foodstuff	*Kcal/gram dry weight*
Carbohydrate	4
Fat	9
Protein	4

Back of these values is the idea that the human body operates simply as a chemical machine No particular apology is required for this assumption now, but when these figures were first derived, the ideas of the vitalists who believed that mere chemistry and physics could never explain the working of the body, were still very powerful and it was a great triumph for the chemists when these figures were actually checked by observations on living human beings

It is possible to build a calorimeter big enough to accommodate a human being, and such a machine makes it possible to measure the heat evolved per given time interval by a patient In a closed system such as this calorimeter any work done by the patient does not affect the outside world as work, for it is converted by friction into heat within the calorimeter This applies to the indispensable vital work such as breathing heart action, kidney action and involuntary muscular motions If we know the amounts and proportions of the three classes of foodstuffs being utilized by the patient, we may compute how much heat he should be producing and compare this with the observations

In order to find out how much of the three different classes of food the patient is oxidizing, we must measure three things To estimate the amount of protein metabolized during the interval we must know how much nitrogen is excreted in the urine All of the urinary nitrogen was once protein, and by assuming the usual conversion factor of 6 25, we may compute the amount of protein metabolized in the time the patient spends in the calorimeter and from this the heat produced from protein metabolism (see page 638) We must also know the amount of oxygen consumed by the patient, and third, we must know the amount of carbon dioxide produced The reasons for wanting these last two items of information will be immediately explained

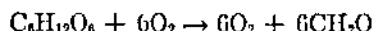
Respiratory Quotients

Different classes of food require varying amounts of oxygen to produce [illegible]

carbohydrates produce more CO_2 per mol of oxygen used, [illegible] [illegible] enough oxygen to oxidize the hydrogen to water and only need [illegible]

they have no molecular oxygen)

The *respiratory quotient* (RQ) of a substance is the ratio of the volume of carbon dioxide produced in the oxidation of a substance to the volume of oxygen used. When we can write the chemical reaction involved, we can compute the RQ. For instance, in the case of glucose, we have the equation

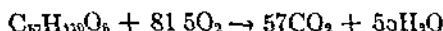


$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6CH_2O$$

Recalling that Avogadro's law states that all gases at standard conditions of pressure and temperature occupy the same volume per mol, we easily see that the ratio

$$\frac{\text{Liters } CO_2 \text{ produced}}{\text{Liters } O_2 \text{ used}} = 6/6 = 1.00$$

For any given fat the theoretical RQ can also be computed. For instance, if we take tristearin we have



$$C_{57}H_{110}O_6 + 81.5O_2 \rightarrow 57CO_2 + 55H_2O$$

So the RQ equals 57/81.5 or 0.699. Corresponding calculations give an RQ of 0.703 for tripalmitin and 0.713 for triolein. Mixed body fats give an RQ of about 0.707. It is generally assumed that a fair average value for the RQ of fats is 0.71.

The computation of the RQ of proteins for their metabolism in the human body is rendered more complicated by the fact that they are not completely oxidized. It seems best to consult an actual experiment. Rubner (3), one of the founders of the science of animal calorimetry, fed an animal dried muscle which had been analyzed chemically. One hundred grams of the dry material contained 5.5 grams of ash. In an experiment calculated by Loewy (3), an animal eliminated, of 100 grams of the organic part of protein, 42.5 grams in the urine and 2.94 grams in the feces. The detailed computations are shown in table 40. The CO_2 produced can be calculated from the remaining C. From this figure and the value of O, again using Avogadro's number, we get

$$RQ = \frac{152.17/44}{137.91/32} = 0.80$$

To estimate the calorific value of dried muscle in the animal body Rubner first burned some in the bomb calorimeter, thus obtaining the total heat value. By similarly burning the urine and feces resulting from the ingestion of 100 grams of the dried muscle and subtracting, Rubner was able to calculate that although the total calorific value of the 100 grams of dried muscle was 534.5 Kcal, 129.77 Kcal was lost in the urine and feces, leaving a fuel value available to the body of 404.73 Kcal. Slight deductions

for the heat present in the protein in its dissolved state but lost on drying, and for the heat of solution involved in dissolving urea and other urinary constituents had to be applied This left 400 06 Kcal as the maximum energy obtainable from 100 grams of the solids of dried meat It is from this that we obtain the 4 Kcal per gram given earlier (page 636) as the calorific value of protein in the body It can be seen that the heat available in the body from protein is about 75 per cent of that available in the bomb calorimeter About 25 per cent of the heat is lost because of the needs of the body to excrete the nitrogen from proteins in suitable forms

If we know the calorific value of protein and know from the nitrogen in the urine the amount of protein metabolized in a given time, we may compute the amount of heat derived from this metabolism of protein, and the amount of oxygen consumed and the amount of carbon dioxide

TABLE 40

Metabolism of protein

	C	H	N	O
Composition of 100 grams of dried muscle	52 38	7 27	16 65	22 68
Urine contains 42 45 grams	9 41	2 66	16 28	14 10
Feces contain 2 94 grams	1 47	0 21	0 37	0 89
Total excreted in urine and feces	10 88	2 87	16 65	14 99
Remainder	41 50	4 40	—	7 69
Allowing for intramolecular water	41 50	3 43	—	—
Oxygen needed for remaining H and C				137 91

produced By subtraction we get the heat, oxygen consumed, and carbon dioxide produced due to the utilization of carbohydrate and fat together If the RQ were the same for carbohydrate and for fat, we should still have the unsolvable problem of trying to determine how much fat and how much carbohydrate the body is metabolizing at a given moment Fortunately they are different, and computation of the non protein heat produced and the RQ enables us to calculate the proportions of fat and carbohydrate being used

As we have seen, the RQ of pure carbohydrate is 1 00 and that of fat is generally assumed to be 0 707 In oxidizing 1 232 grams of carbohydrate, one liter of oxygen is consumed and 5 047 Kcal of heat is produced One liter of oxygen oxidizes 0 502 grams of fat with the production of 4 686 Kcal of heat Mixtures of carbohydrate and fat in various proportions give RQ values intermediate between 0 707 and 1 00 and caloric values between 5 047 and 4 686 The amounts of carbohydrate and fat and the heat produced in a mixture requiring one liter of oxygen corresponding to various RQ can be computed, and are shown in Table 41a

This table is based on one published by Lusk (3) which is in turn a modification of one published by Zuntz and Schumberg A modification of

TABLE 41a

Calculated Relation between Amounts of Carbohydrate and Fat used at Various RQ, for the Consumption of One Liter of Oxygen

NONPROTEIN RQ	EQUIVALENTS OF ONE LITER O_2 USED		
	Fuel		Energy
	Carbohydrate	Fat	
	grams	*grams*	*Kcal*
0.707	0.000	0.502	4.686
0.71	0.013	0.497	4.690
0.72	0.055	0.480	4.702
0.73	0.097	0.463	4.714
0.74	0.139	0.445	4.727
0.75	0.181	0.428	4.739
0.76	0.223	0.411	4.751
0.77	0.265	0.394	4.764
0.78	0.307	0.377	4.776
0.79	0.349	0.360	4.788
0.80	0.391	0.343	4.801
0.81	0.433	0.326	4.813
0.82	0.475	0.308	4.825
0.83	0.517	0.291	4.838
0.84	0.559	0.274	4.850
0.85	0.601	0.257	4.862
0.86	0.643	0.240	4.875
0.87	0.685	0.223	4.887
0.88	0.727	0.206	4.899
0.89	0.769	0.188	4.911
0.90	0.812	0.171	4.924
0.91	0.854	0.154	4.936
0.92	0.896	0.137	4.948
0.93	0.938	0.120	4.961
0.94	0.980	0.103	4.973
0.95	1.022	0.086	4.985
0.96	1.064	0.069	4.998
0.97	1.106	0.051	5.010
0.98	1.148	0.034	5.022
0.99	1.190	0.017	5.035
1.00	1.232	0.000	5.047

Lusk's table is reproduced in most textbooks dealing with this subject Unfortunately this table contains many errors, probably computational, amounting in some cases to as much as 23 per cent The errors are probably too small to be of practical importance, but have been corrected here for the sake of consistency

If we let c represent the number of grams of carbohydrate in the mixture being oxidized at any given RQ, and f represent the number of grams of fat, the mixture requires, by hypothesis, exactly one liter of oxygen, comprised of the amounts $c/1.232$ and $f/0.502$, and we have

$$c/1.232 + f/0.502 = 1$$

The carbon dioxide produced will be $c/1.232$ and $0.707\ f/0.502$ The sum of these two quantities divided by the oxygen used, which is unity, gives the RQ, so we have

$$c/1.232 + 0.707\ f/0.502 = RQ$$

If these two equations are solved, expressions are obtained for c and f in terms of RQ

$$c = 4.2048\ (RQ - 0.707) \qquad f = 1.7133\ (1 - RQ)$$

The values of c and f thus computed are shown in Table 41 The calorific value of one liter of oxygen for such mixtures is

$$5.047\ c/1.232 + 4.686\ f/0.502$$

shown in the right hand column

More recent analyses of human fat lead to values somewhat different from those in Table 41a Cathcart and Cuthbertson (1a) analyzed the fat of the panniculus adiposus abdominalis from seven subjects and found that 0.522 gram, utilizing one liter of oxygen, gives a calorific value of 4.749 Kcal A composite result from the analyses of fat from skeletal muscle and liver, which Cathcart and Cuthbertson believe to be more representative of the fat being oxidized by the fasting patient, indicated that 0.516 gram of fat utilized one liter of oxygen with the production of 4.735 Kcal of heat Acceptance of these results would modify the figures in Table 41a, especially in the upper part where the mixtures being utilized consist mainly of fat See Table 41b

From this table it will be seen that if we deduct the heat produced by the metabolism of protein, a measurement of the RQ enables us to compute the proportions in which fat and carbohydrate are being utilized at the same time When all this information was used, it was found that a patient ... calorimeter produced heat consumed oxygen, and produced carbon

confident that the phenomena of the body, although compl... not outside the ordinary laws of physics and chemistry This proof is of ... importance to us today but the technique, somewhat modified,

TABLE 41

Calculated amounts of carbohydrate and fat consumed and heat produced during utilization of one liter of oxygen as related to observed values of the nonprotein RQ. Calculated from the values of Cathcart and Cuthbertson

NONPROTEIN RQ	EQUIVALENT OF ONE LITER OF OXYGEN		
	Fuel		Energy
	Carbohydrate	Fat	
	grams	*grams*	*Kcal*
0.718	0.000	0.516	4.735
0.72	0.009	0.512	4.737
0.73	0.052	0.494	4.737
0.74	0.096	0.478	4.748
0.75	0.140	0.457	4.759
0.76	0.183	0.439	4.770
0.77	0.227	0.421	4.781
0.78	0.271	0.403	4.793
0.79	0.315	0.384	4.804
0.80	0.358	0.366	4.815
0.81	0.402	0.348	4.826
0.82	0.446	0.329	4.837
0.83	0.489	0.311	4.848
0.84	0.533	0.293	4.859
0.85	0.577	0.274	4.870
0.86	0.620	0.256	4.881
0.87	0.664	0.238	4.892
0.88	0.708	0.220	4.903
0.89	0.751	0.201	4.914
0.90	0.795	0.183	4.925
0.91	0.839	0.165	4.936
0.92	0.882	0.146	4.947
0.93	0.926	0.128	4.958
0.94	0.970	0.110	4.970
0.95	1.104	0.091	4.981
0.96	1.057	0.073	4.992
0.97	1.101	0.055	5.003
0.98	1.145	0.037	5.014
0.99	1.188	0.018	5.025
1.00	1.232	0.000	5.036
			5.047

which was brought up earlier—namely, how efficient is the human body in converting the energy of foodstuffs into work?

Mechanical Efficiency of the Human Body

Experiments can be carried out in which a man does work inside a calorimeter. In one accurately controlled experiment the patient did work by pumping the pedals of a stationary bicycle, and the work done as well

as the heat produced were recorded During the performance of the mechanical equivalent of some 550 Kcal of work 4550 Kcal of heat were produced Both the heat and the work must have come from the oxidation of foodstuffs, so the total input to the human machine in this experiment was about 5100 Kcal of which 10 8 per cent was manifested as work This is a little better than the efficiency of a locomotive, but not as good as a good diesel, and not at all to be compared with the mechanical efficiency of a battery motor combination If the body does indeed function as a chemical cell, what is the explanation of such a low efficiency?

In the first place the body must keep itself alive, which is a more complicated requirement than any ordinary electric cell has to meet The breathing, pumping of the heart perspiration, and possibly other vital functions had to go on during the work period not merely as usual but at a greatly accelerated rate This must have used up a good deal of the energy the body produced in the form of internal work and caused this part of the work to be converted into heat Thus this work does not appear in our tabulation of the work performed But the most important factor in lowering the apparent efficiency of the body is the conversion of work into heat in overcoming the internal friction of the muscles themselves The muscle tissue is very viscous and it has been calculated that when the body is producing work at a high rate as in riding a bicycle, or running a race, the greater part of the energy of the foodstuffs being oxidized is utilized to overcome this internal friction and is thus converted into heat Therefore it would not be surprising if the individual whose performance was just discussed really converted a high percentage of the energy of his foodstuffs into mechanical energy but most of this energy was internally degraded into heat In fact, we are sure this is exactly what did happen

Zuntz, followed by later workers (3), has attempted to measure the efficiency with which the muscles of the human body can convert the energy of foodstuffs into work by comparing the heat output of the body while working without any external load with the total energy output (heat plus work) when working under load Even this probably does not make nearly adequate allowance for the added burden on the heart and lungs during work, and the energy consumed in overcoming internal friction, so that we are getting a minimum estimate of efficiency from such measurements Nevertheless, one subject, studied while walking up a five degree slope achieved a mechanical efficiency of over 40 per cent for a short time, which puts him in a class with the best internal combustion engines we can construct The actual efficiency in the conversion of the energy of the fuels to work is probably much higher in the human body than in a diesel However, we are forced to judge by the only thing we can measure, namely the work done on the external environment In this respect the body and a diesel engine are mechanically about on a par

Cost of Work from Various Sources

There is another aspect to comparisons of the efficiency of the human body with that of other chemical cells and with heat engines, and that is the economic one. How much does it cost to produce one Kcal. of work by the various methods? When we examine this we find that the human body, because of the cost of its fuels, can not compete even with the steam locomotive although it does produce work more cheaply than the ordinary dry cell combined with an electric motor.

Books on thermodynamics do not take any account of the cost of producing work by various methods, since they take it for granted that the engineer in charge of a plant has sense enough to get his heat by burning the cheapest fuel locally available and knows that electric cells such as the dry cell are far too expensive to use for the large scale production of work. But it is instructive for our purposes to make some comparisons of the cost of work from different systems and this is done in table 42. In making these calculations it has been assumed that sheet zinc costs 50 cents per pound (the cost of other components of the dry cell being ignored), cane sugar (a good fuel for the human body) something less than 10 cents per pound, diesel oil 13.4 cents per gallon, and coal $25 per ton. The mechanical efficiency of the diesel and of the human body have been estimated at 40 per cent, the steam locomotive at 8 per cent, the dry cell motor combination at 80 per cent (which is probably too high).

TABLE 42

Costs (in cents) of 1000 Kcal. of energy in the form of work as obtained from different sources

MATERIAL (FUEL)	METHOD	COST OF 1000 KCAL.
		cents
Zinc metal	Oxidation in dry cell plus electric motor	130.0
Sugar	Enzymatic oxidation in the human body	13.0
Coal	Steam locomotive	4.06
Fuel oil	Oxidation in diesel engine	0.86
(Coal)	(Electrochemical oxidation in hypothetical $C + O_2$ cell)	0.45

In addition the table contains figures for the production of work from coal by a process which possesses theoretical interest, but has never been realized practically. This involves the direct use of carbon and oxygen in a cell making use of the reaction



$$C + O_2 \rightarrow CO_2$$

Such a cell, if it could be constructed, would be the most efficient method

of getting work from coal (6) However, although many clever inventors such as Edison have worked on this problem it still seems far short of solution and the results are included here merely for use in comparisons

No figures are given for the work obtainable from radioactive materials for although the yield per gram of material is hundreds of thousands times as great as that from coal the earth s supply of fissionable elements appears to be so limited that it seems likely that such energy may never be of great economic importance except for special applications in warfare For other uses we shall have to depend upon the energy from the sun as stored up in plants by photosynthesis (2) Coal is of course simply fossilized plant material

Unless methods can be devised for utilizing solar energy as by growing starchy plants in the tropics, fermenting the carbohydrate to alcohol and burning it in efficient internal combustion engines we may presumably be reduced someday when our coal and oil are all gone to the use of work from animal muscle The horse is a cheaper source of work than the human because the horse can utilize hay and other very cheap sources of carbo hydrates But we should remember that even without the aid of the work of domestic animals energy furnished by human muscles has in the past performed feats like erecting the temple of Chichén Itzá the Colossi of Memnon and the Pyramids at Giza

Indirect calorimetry Since as we have mentioned the rate at which a resting patient produces heat has clinical significance it is convenient to be able to estimate this without having to measure the heat directly by putting the patient inside an unwieldy calorimeter big enough to accom modate human beings with its elaborate insulation water circulation to collect the evolved heat oxygen supply carbon dioxide and water collect ing systems and forced ventilation From the results of the early work on animal calorimetry it became clear that the heat evolved could be esti mated with sufficient accuracy without measuring it directly From the above discussion it will be seen that if the patient were metabolizing only fat and carbohydrate measurement of the RQ and the rate of oxygen consumption would enable us to calculate the rate at which he was evolving heat If we knew the ratio in which fat and carbohydrate were being me tabolized we should know also the RQ and consequently merely measuring the rate of oxygen consumption would be enough

This is all very well, but the chances are that the patient is also metabo lizing some protein and to ascertain this measurements of urinary nitrogen would be needed The problem sounds rather complicated But for once nature gave the scientist a lucky break The heat output when the patient is resting proves to be the measurement which is of most clinical importance and it was discovered that the RQ of such a resting patient provided he was not digesting a meal was 0 82 (within limits of 5 per cent either way)

which is not very different from the RQ of protein The amount of protein being utilized in such a post absorptive state is probably small compared to the amount of mixed fats and carbohydrates thus although the heat production per liter of oxygen is less for protein (4 485 Kcal), we may without serious error assume that an RQ of 0 82 implies that the patient is utilizing for each liter of oxygen consumed 0 416 grams of carbohydrate and 0 329 grams of fat and is thus producing 4 818 Kcal of heat per liter of oxygen (see table 41) This fortunate circumstance enables the heat output of the resting post absorptive patient to be estimated merely from a measurement of the rate at which oxygen is being consumed The apparatus for doing this is much simpler and easier to use than the elaborate and expensive calorimeters we have been discussing Basically we need only a means of measuring the oxygen consumption For patients doing work where the RQ can not be assumed but must be measured the expired air must be collected in a spirometer and analyzed For mountain climbing experiments for instance an apparatus called the Douglas bag was devised in which the subject collects his expired air in a bag carried on his back The oxygen consumed can be calculated from the volume oxygen content and nitrogen content of the expired air The carbon dioxide can be calculated from the volume and CO_2 content From these data the RQ can be computed and analysis of the urine enables the amount of protein utilized to be estimated

Suppose we consider the following data for the 24 hour value of a normal person

Carbon dioxide eliminated	306 liters
Oxygen consumed	350 liters
Total nitrogen of the urine	11 grams

We assume that the average protein molecule contains 16 per cent nitrogen and therefore the conversion factor of nitrogen to protein is 6 25 The metabolism of an amount of protein equivalent to one gram of urinary nitrogen will require 5 92 liters of oxygen and will yield 4 75 liters of carbon dioxide Multiplying we find that the elimination of 11 grams of nitrogen in the urine shows 52 25 liters of carbon dioxide were produced due to protein oxidation and 65 12 liters of oxygen were used up during the oxidation of protein

If we subtract these values from the totals we find that 253 8 liters of carbon dioxide were produced by the oxidation of fats and carbohydrates, and 284 9 liters of oxygen were used in the process The non protein RQ is thus

$$\frac{253\ 8}{284\ 9} = 0.89$$

In clinical practice it is not usually necessary to measure the RQ It is only necessary to be assured that the patient is in a so called basal condition —that is not working or actively digesting a meal recently consumed and we may then assume without serious error that the RQ is 0 82 which means that each liter of oxygen being utilized provides energy of 4 848 kcal

Utilizing these assumptions it is therefore possible to convert the oxygen consumption for a given time into the corresponding value of calories liberated without knowing the other data which would be required of indirect calorimetry not in the basal state (carbon dioxide production and urinary nitrogen)

Basal Metabolic Rates

The rate at which the human subject is utilizing oxygen without the influence of food or muscular work is the basis for computing the basal metabolic rate (BMR) It is assumed that the BMR measures the energy required for the fundamental processes which must go on in the body to maintain life In order to measure BMR one must use a subject resting but awake, who has had no food for 14 to 18 hours and has indulged in no muscular activity for approximately half an hour

This basal metabolism is not quite equivalent to the minimal metabolism which is the mere maintenance of life as is shown by the fact that during sleep the rate of oxygen consumption measured is somewhat lower than during the so called basal state

Measurement of the basal metabolic rate has clinical importance and may be briefly described here The patient having taken no food since the preceding evening comes to the laboratory before breakfast with a minimum expenditure of work, lies in complete muscular and mental repose for about 30 minutes, and then breathes pure oxygen from a closed system through a facial mask or some similar device

The apparatus used consists of a device which contains oxygen, and records changes in its volume The patient is connected to the apparatus in such a way that the air returned by the patient passes through soda lime which removes the CO_2 produced From the rate of O_2 consumption the rate of heat production is computed as described above

There is a close correlation between the insensible loss of weight from the body (by evaporation of water and loss of CO_2) and the BMR (4) so that a measurement of this insensible weight loss by use of a good balance large enough to support a human being enables the BMR to be calculated quite readily BMR determinations on many normal persons have enabled us to establish accurate standards for the BMR (5)

In the early days of animal calorimetry Rubner proposed that the rate of heat liberation was proportional to the surface area of the body, and

elaborate measurements of the surface area were made and nomograms and other aids in calculating the surface area of the body were devised However, the relationship is only a rough one The basal metabolism is more nearly proportional to the three fourths power of the body weight

The normal BMR is now calculated from measurements on the patient, usually weight and sitting height, allowing for age and sex The normal BMR to be expected varies somewhat with the sex of the patient, with age, condition of nutrition, and so on

Various internal glands—especially the thyroid—affect the BMR If the normal functioning of the thyroid is decreased, it may lower the BMR by as much as 25 per cent and occasionally even more Correspondingly, an overactive thyroid may increase the BMR as much as 40 per cent above normal Other endocrine glands, such as the pituitary, may also cause disturbances of the BMR It is also increased by fever, dyspnea, leukemia, and polycythemia The BMR is decreased in starvation, hypothyroidism, and Addison's disease

There is a certain variation shown in the BMR in persons in normal health It is generally considered that this normal variation amounts to ±10 per cent of the mean value as computed from the usual standards Unless deviations exceed this, they are not considered indicative proof of an abnormal condition

Specific Dynamic Action

The ingestion of food raises the metabolic rate This is true of all foods, but there is a more pronounced effect from protein than from fat or carbohydrate This tendency to increase the rate of oxidation is called the specific dynamic action (SDA) It is not due to the increased muscular activity of the digestive system for it is not produced by feeding non-digestible substances or substances which yield no calories as cellulose and meat extracts, although these do stimulate the activity of the gastrointestinal musculature

The Effect of Muscular Work

The operation of the muscles of the body is responsible for the liberation of a considerable amount of the body's heat The human body at rest may be compared to an idling motor Heat is being produced, whether it is needed or not, at about the rate of 80 Kcal per hour (1) Various forms of activity increase this, depending on the degree of exertion For instance, a patient *sitting and reading produces about 105 Kcal per hour* For other forms of exertion the following results have been reported typewriting, 140 swimming, 400 to 500, running, 570 sawing wood, 480 The body is capable of producing heat at a more rapid rate than this (over 1000 Kcal

per hour) when the exercise is heavy and performed at such a rate that most of the energy appears as heat, as in rapid stair climbing or a 100 yard dash but effort at such rates can not be sustained

The Daily Requirement of Calories

Computations, based upon the basal metabolism of normal subjects and estimates of their normal requirements in the way of extra energy, have led to a common estimate of about 2500 Kcal per day as the average fuel requirement of a sedentary man With increased activity, very much more is of course required A bicycle rider, riding a six day race, needs about 10,000 Kcal per day It has already been noted that most of the calories of the diet appear as heat and only a fraction as work

REFERENCES

1 Adolph E F *Physiology of Man in the Desert* New York N Y Interscience Publishers 1947

1a Cathcart E P and Cuthbertson D P The composition and distribution of the fatty substances of the human subject J Physiol 72 349-360 1931

2 Hoyle F *The Nature of the Universe* Oxford Eng Blackwell 1951

3 Lusk G *The Science of Nutrition* Philadelphia and London W B Saunders Co 1928

4 Newburgh L H and Johnston M W The insensible loss of water Physiol Rev 22, 1 18 1942

5 Peters J P and Van Slyke D D *Quantitative Clinical Chemistry* 2nd ed t Baltimore Md The Williams & Wilkins Co 1946

6 Thompson M D K *Theoretical and Applied Electrochemistry* New York N Y Macmillan Co 1925

CHAPTER 18

Excretion and Some of Its Disturbances

Carbon dioxide is produced continuously by the human body at a rate varying with its mass and activity. The removal of CO_2 by the lungs has already been described. Certain other substances are produced by the body with equal consistency. We have seen that urea, uric acid, and creatinine are end products of normal and indispensable metabolic processes. Similarly, the formation of SO_4^{--} and HPO_4^{--} from S and P of foodstuffs is as inevitable as the production of CO_2. Water is also a metabolic product but not in amounts adequate to meet the obligations of the excretory mechanisms. The elimination of CO_2 by the lungs, of solutes by the kidney, and of metabolic heat by both these organs and by the skin, all require the output of water in excess of the amount produced by metabolic processes. The kidney is the chief organ for the elimination from blood and body fluids of excess solids with the notable exception of the degradation products of hemoglobin, which leave the body as bile pigment derivatives chiefly by way of the intestinal tract. In renal failure the intestine and even the skin perform excretory functions which are in those organs insignificant during health.

The kidney is, however, no simple sluiceway through which a stream of water indiscriminately bears the effluvia of metabolism. The kidney, more than any other organ, regulates the total osmotic pressure of all body fluids and participates in the regulation of the concentration of each and every soluble and diffusible component of the extracellular fluid—the internal environment in which our component cells live and operate. Let us select a single example of this precise regulatory function. The kidneys maintain the concentration of cations in plasma at an excess of 25 to 27 mEq per liter above the sum of the concentration of non-volatile anions. This excess of cations, by the law of electroneutrality, retains in the plasma an equivalent concentration of HCO_3 (the only available volatile anion) which is thereby maintained at 25 to 27 mEq per liter. Meanwhile the physiological control of respiration is keeping the plasma H_2CO_3 at a con

centration between 1 25 and 1 35 mEq per liter Filling in these values, and the physiological pK of H_2CO_3, we can set up the Henderson Hasselbalch equation for the bicarbonate system in plasma

$$pH = 6\ 10 + \log \frac{26}{1\ 30} = 7\ 4$$

Thus the actual maintenance of the pH of the plasma and interstitial fluid at its constant value of 7 4 depends upon the opposed actions of lungs and kidneys (28) The mechanisms of acid base regulation by the kidney will be dealt with in a later section This example was selected to illustrate the *regulatory* function of the kidney, which is a refinement of the excretory function In brief, the kidney responds to high concentrations of many substances in the plasma by excreting them at a more rapid rate By so doing, the kidney not only regulates concentrations of individual ions and molecules, but also stabilizes the pH and osmotic pressure of blood plasma and indirectly of other body fluids

There are three steps in the formation of urine by an individual nephron (a) *filtration* in the glomerulus, (b) *absorption* of water and certain solutes in the tubules, and, (c) *excretion* of water and certain solutes by the tubule cells The amount and composition of the urine formed by these steps is dependent upon physical forces, such as pressure and velocity of blood, as well as upon the chemical composition of the blood Glomerular filtrate contains the diffusible components of the blood plasma in concentrations similar to those in the water of plasma Work is done by the kidney against osmotic pressure in the conversion of glomerular filtrate into the more concentrated urine The difference in osmotic pressure between blood and urine, as estimated from freezing point determinations, averages about 25 atmospheres For each liter of urine this means a minimal energy requirement of 25 liter atmospheres or 0 6 Kcal, the equivalent of the total combustion of 0 15 grams of glucose The actual metabolic activity of the kidney is many times greater than this minimal figure The less the glomerular filtrate undergoes concentration as it passes through the renal tubules, the less the energy required The process of concentration is not uniform for the different urinary solutes Different substances are concentrated to different degrees The SO_4^{--} ion is ordinarily concentrated about 90 times, the Cl^- ion only twice, the Na^+ ion has approximately the same concentration in urine as in blood plasma while glucose is normally completely reabsorbed in the tubules and does not appear in any appreciable amount in the urine

Physicians, now and throughout the recorded history of medicine, consider study of the urine an integral and important part of the physical examination of the patient To be sure, much of the attention given to

urinoscopy in ancient and medieval medicine depended upon the fact that urine was the only body fluid easily and painlessly available, and some of the archaic diagnostic and prognostic manipulations of the specimen were heavily tainted with sympathetic magic We still find it diagnostically useful to examine the urine, although we now can intubate the vena cava and cannulize the cisterna magna

The purposeful collection of urinary specimens over a measured time is a matter of some importance The 24 hour period is usually most convenient in all types of long term metabolic studies, as for example in the diagnosis of hyperparathyroidism (see page 591) Shorter metabolism periods suffice for most of the renal function tests to be described later Reducing sugars are more likely to be detected from one to two hours after a meal, chorionic gonadotrophins in the concentrated early morning urine, albumin when physical activity is resumed after a night's sleep, other instances of timing will appear as we go along When quantitative methods are to be applied, timing must be exact

Another important and often neglected factor in the outcome of urine examinations is proper preservation of specimens One hour's standing in an overheated utility room or laboratory can turn a glycosuric urine specimen from strongly Benedict positive to Benedict negative The microorganisms which catalyze this fermentation of sugar are ubiquitous, and often inhabit the urinary bladders of diabetics With bacterial multiplication, urines may develop heat coagulable proteins, cells, casts, and other microscopically visible components will decompose, and as a result of changes in pH crystals may deposit Refrigeration is usually adequate to control undesirable chemical changes Toluene or chloroform, one ml per liter, will temporarily restrain the multiplication of yeasts and bacteria Formaldehyde is an almost perfect preservative for cells and casts, but in high concentrations interferes with some chemical tests

PHYSICAL CHARACTERISTICS OF THE URINE

Considerable information of diagnostic value can be obtained by direct observation of a urine specimen, aided only by a few simple instruments The normal amber *color* of urine is the result of the presence of several normal pigments, which will be discussed in a later section The depth of the amber color is a rough index of the extent to which the urine has been concentrated, which in turn is dependent to some extent on the patient's state of hydration Pathological or unusual coloring of the urine may result from *drugs, bile pigments, blood pigments, homogentisic acid, melanin*, or oxidation products of phenols The presence of porphyrins in abnormal amount is not always indicated by abnormal color of the urine, but yields a red fluorescence under filtered ultraviolet light Normal urine

is clear and transparent, except for a slight and slowly gathering sediment of epithelial cells and *mucoprotein* from the urinary tract (this sediment is the "nubecula" of the old time urinoscopists who made quite a fuss about it). However, the urine of a healthy person may contain a precipitate of calcium and magnesi
line This may result f
are The *urine of horse*
During the time of ac
and may become basic or alkaline urine may be simply the result of enthusiastic use of sodium bicarbonate for its supposed therapeutic effects. Any urine specimen on standing in a warm place may support the growth of microbes which hydrolyze urea to ammonium carbonate and may thereby turn alkaline and deposit phosphates. Phosphatic sediments will clear promptly with acidification and are usually without direct pathological significance. All sediments other than those already mentioned are abnormal (not necessarily pathological) and should be investigated. Certain crystals, such as calcium oxalate and uric acid, are often seen in normal urine under the microscope but do not occur normally in amounts sufficient to cause visible turbidity unless the urine is refrigerated. Abnormal turbidity occurs from pus, blood, excessive mucus, and less commonly from insoluble compounds. The odor of urine yields little useful information unless it be notably putrid, which in a fresh specimen would indicate active bacterial contamination within the urinary tract or a vesicointestinal fistula. The normal odor of fresh urine is contributed chiefly by aromatic acids such as phenylacetic and by traces of mercaptans contributed by vegetable foodstuffs such as onions and asparagus. On standing urines acquire an ammoniacal odor from microbial conversion of urea.

The daily *volume* of urine of adults living in the United States is usually between 0.8 and 1.6 liters. Less than 0.5 liters is normally accumulated in the bladder during the night's sleep. The output is of course subject to great variation from differences in fluid intake and in sweating. The increased urine output in cold weather is proverbial. By purposeful increase of fluid intake a normal person can raise his output to 8 liters or more in a day. Further variations arise from differences in diet: proteins form urea which obligates additional water for its excretion; the caffeine of a cup of coffee has a measurable diuretic effect. Emotional states affect urinary volume in ways which are not uniform among different individuals. The urine volume is characteristically increased in certain diseases, including diabetes mellitus, diabetes insipidus, most chronic nephritides, and during convalescence from acute infections. The volume is characteristically decreased during fevers, following loss of fluids (see Chapter 15), in acute nephritis, and in the majority of circulatory diseases.

The normal 24 hour urinary output of 0.8 to 1.6 liters can be approximated by considering the output as one ml per minute. This rate is subject to continuous and wide variation. No definite limits set off normal rates of output from *diuresis*, or increased rate on one hand, or from *antidiuresis*, or less commonly *oliguresis*, meaning decreased rate on the other. These terms are applied to transitory changes in rate of urine excretion, as measured by volume output. *If the changes are of long duration*, an excretion of more than 2 liters per 24 hours is called *polyuria* and one of less than 0.5 liters, *oliguria*. *Anuria* refers to periods where no urine is formed at all, regardless of duration.

Increased water content of the body stimulates increased urine volume output, acting through the posterior pituitary mechanism discussed in Chapter 15. The presence of osmoreceptor cells in the central nervous system is postulated. *These cells are stimulated by lowered osmotic pressure* (increased water content) of the blood of the internal carotid circulation, and respond by inhibiting the secretion of the antidiuretic hormone by the *pars nervosa* of the hypophysis. The action of the antidiuretic hormone is to promote absorption of water by the renal tubular cells. When this action is inhibited completely as a result of high fluid intake or in diabetes insipidus, the water content of the urine is comparable to that of *glomerular filtrate*, *and the specific gravity of the urine is 1.010 or less*.

The *specific gravity* of the urine is usually measured with a simple hydrometer. This is accurate enough for all purposes except the more refined investigative work, provided the hydrometer has been properly calibrated, and is periodically checked against solutions of known specific gravity. The temperature correction, 0.001 added for each 4°C above the calibration *temperature of the hydrometer, should always be included*. *In the interpretation* of specific gravities in clinical diagnosis, it is advisable to make another correction for large amounts of proteins, if such be present, by subtracting 0.003 for each one per cent of protein found. Most normal urine specimens have specific gravities between 1.008 and 1.030 and most 24 hour collections between 1.016 and 1.022, but variations beyond these limits occur. It can *not* be too strongly emphasized that variation of specific gravity *throughout the 24 hours is characteristic of normal regulatory* function of the kidney, and that fixation of specific gravity means deterioration of such functional ability. For this reason, most single specific gravity measurements are not contributory to diagnosis, while repeated measurements are informative. More of this matter will come up later. The specific gravity of the urines of young infants tends to be low by adult standards, and does not increase in severe dehydration to the extent observed in the adult (22). The ability to produce a concentrated urine also decreases in

is clear and transparent, except for a slight and slowly gathering sediment of epithelial cells and mucoprotein from the urinary tract (this sediment is the "nubecula" of the old time urinoscopists, who made quite a fuss about it). However, the urine of a healthy person may contain a precipitate of calcium and magnesium phosphates if the specimen happens to be alkaline. This may result from an alkaline ash diet, as most vegetarian diets are. The urine of horses and cattle is usually turbid with such phosphates. During the time of active gastric secretion, the urine becomes less acid and may become basic, or alkaline urine may be simply the result of enthusiastic use of sodium bicarbonate for its supposed therapeutic effect. Any urine specimen, on standing in a warm place, may support the growth of microbes which hydrolyze urea to ammonium carbonate, and may thereby turn alkaline and deposit phosphates. Phosphatic sediments will clear promptly with acidification, and are usually without direct pathological significance. All sediments other than those already mentioned are abnormal (not necessarily pathological) and should be investigated. Certain crystals, such as calcium oxalate and uric acid, are often seen in normal urine under the microscope, but do not occur normally in amounts sufficient to cause visible turbidity unless the urine is refrigerated. Abnormal turbidity occurs from pus, blood, excessive mucus, and less commonly from insoluble compounds. The odor of urine yields little useful information, unless it be notably putrid, which in a fresh specimen would indicate active bacterial contamination within the urinary tract or a vesicointestinal fistula. The normal odor of fresh urine is contributed chiefly by aromatic acids such as phenylacetic and by traces of mercaptans contributed by vegetable foodstuffs such as onions and asparagus. On standing, urines acquire an ammoniacal odor from microbial conversion of urea.

The daily *volume* of urine of adults living in the United States is usually between 0.8 and 1.6 liters. Less than 0.5 liters is normally accumulated in the bladder during the night's sleep. The output is of course subject to great variation from differences in fluid intake and in sweating. The increased urine output *in cold weather* is proverbial. By purposeful increase of fluid intake, a normal person can raise his output to 8 liters or more *in* a day. Further variations arise from differences *in diet*: proteins form urea which obligates additional water for its excretion; the caffeine of a cup of coffee has a measurable diuretic effect. Emotional states affect urinary volume in ways which are not uniform among different individuals. *The urine volume is characteristically increased in certain diseases including diabetes mellitus, diabetes insipidus, most chronic nephritides, and during convalescence from acute infections.* The volume is *characteristically decreased during fevers, following loss of fluids (see Chapter 15), in acute nephritis, and in the majority of circulatory diseases.*

The normal 24 hour urinary output of 0.8 to 1.6 liters can be approximated by considering the output as one ml. per minute. This rate is subject to continuous and wide variation. No definite limits set off normal rates of output from *diuresis*, or increased rate on one hand, or from *antidiuresis* or, less commonly, *oliguresis*, meaning decreased rate on the other. These terms are applied to transitory changes in rate of urine excretion as measured by volume output. If the changes are of long duration, an excretion of more than 2 liters per 24 hours is called *polyuria* and one of less than 0.5 liters, *oliguria*. *Anuria* refers to periods where no urine is formed at all, regardless of duration.

Increased water content of the body stimulates increased urine volume output, acting through the posterior pituitary mechanism discussed in Chapter 15. The presence of osmoreceptor cells in the central nervous system is postulated. These cells are stimulated by lowered osmotic pressure (increased water content) of the blood of the internal carotid circulation, and respond by inhibiting the secretion of the antidiuretic hormone by the *pars nervosa* of the hypophysis. The action of the antidiuretic hormone is to promote absorption of water by the renal tubular cells. When this action is inhibited completely, as a result of high fluid intake or in diabetes insipidus, the water content of the urine is comparable to that of glomerular filtrate, and the specific gravity of the urine is 1.010 or less.

The *specific gravity* of the urine is usually measured with a simple hydrometer. This is accurate enough for all purposes except the more refined investigative work, provided the hydrometer has been properly calibrated, and is periodically checked against solutions of known specific gravity. The temperature correction, 0.001 added for each 4°C. above the calibration temperature of the hydrometer, should always be included. In the interpretation of specific gravities in clinical diagnosis, it is advisable to make another correction for large amounts of proteins, if such be present, by subtracting 0.003 for each one per cent of protein found. Most normal urine specimens have specific gravities between 1.008 and 1.030 and most 24 hour collections between 1.016 and 1.022, but variations beyond these limits occur. It can not be too strongly emphasized that variation of specific gravity throughout the 24 hours is characteristic of normal regulatory function of the kidney, and that fixation of specific gravity means deterioration of such functional ability. For this reason, most single specific gravity measurements are not contributory to diagnosis, while repeated measurements are informative. More of this matter will come up later. The specific gravity of the urines of young infants tends to be low by adult standards and does not increase in severe dehydration to the extent observed in the adult (22). The ability to produce a concentrated urine also decreases in

elderly people even in the absence of renal disease Various "coefficients' have been proposed by which the total solids of the urine may be presumably calculated from the specific gravity These calculations are in error whenever the urine varies from a fixed ratio of its chief components, urea and NaCl Since such variations are inherent in normal renal function, the arbitrary conversion of known specific gravity into doubtful total solids adds no information and may introduce confusion

ACIDITY AND ALKALINITY

The limits of pH observed in human urine are 4 5 and 8, with the mode at about pH 6 The chief sources of the usual acidity of human urine are proteins and phospholipids of the diet, which yield sulfuric and phosphoric anions as end products of their metabolism The cations required to balance these anions decrease the supply of cations available in the plasma for carrying CO_2 in the form of HCO_3^- Excretion of alkali and alkaline earth cations associated with SO_4^{--} and with HPO_4^{--} would proceed at a rate faster than their supply to the body, and would cause depletion of cations were it not for two important conservative mechanisms functioning in the kidney (a) the excretion of an acid urine, and, (b) the formation and excretion of NH_4^+

The normal daily output of acid in the urine, as measured by Folin's (9) method of titration, is from 15 to 40 mEq To this should be added the acid neutralized by ammonia formed in the kidney and excreted as ammonium salts, this normally amounts to 40 to 60 mEq more In acidotic states, such as diabetic ketosis, very much higher values for the sum of these two acid fractions are observed, up to 700 mEq in very severe acidosis This increase in acidity in diabetic ketosis is largely accounted for by the ketone acids In normal urine, organic acids make up less than 4 per cent of the titratable acidity At the pH of urine, free sulfuric and phosphoric acids are not present, but are excreted in the form of acid salts

Herbivorous animals and human vegetarians ingest salts of organic acids—malates, citrates, acetates, tartrates—in much greater amounts than in the normal mixed diet of the human The acid radicals of such organic salts are oxidized eventually to CO_2 and eliminated via the lungs The cations of the salts may be in excess of the amount needed to neutralize the acid products of metabolism If so, the urine will be alkaline This explains the increase in alkalinity of human urine following ingestion of acid fruits or fruit juices

The mechanism whereby the kidney excretes urine which is definitely more acid or more alkaline than the blood plasma is not fully understood although the problem has been subjected to much analysis The urine exaggerates pH changes of the plasma If the plasma pH is above 7 4, the

pH of the urine will be still higher, similarly, with plasma pH below 7 4, the pH of the urine will be still lower Much fluctuation in urinary pH occurs without measurable change in plasma pH Pitts (28) postulates ionic exchange in the renal tubule cell, H^+ leaving the cell for the lumen of the tubule in exchange for Na^+ or other metallic cations The H^+ is considered to arise within the cell by a process involving the oxidation of a metabolite with production of CO_2, from which H_2CO_3 is formed, under the catalysis of carbonic anhydrase The H_2CO_3 is the source of the H^+ exchanged for Na^+, and $NaHCO_3$ leaves the cell and enters the venous blood Note the similarity of this mechanism with that proposed for the formation of gastric HCl (see page 447)

Approximately 60 per cent of the ammonia formed by the renal tubule cells arises from the glutamine of the plasma by the action of *glutaminase* which converts glutamine to ammonia and glutamic acid Tracer studies have shown that the rest of the ammonia is derived from other amino acids with only a negligible amount from urea Acidity of the urine is the *stimulus* for ammonia production, acting *locally* on the tubule cell In extreme acidosis, 75 per cent of the acid excreted may be combined with ammonia, effecting a considerable saving of essential metallic cations

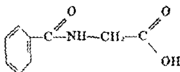
COMPOSITION OF NORMAL URINE

The single solute usually present in greatest amount in human urine is *urea*, the major end product of protein metabolism in mammals The manner of its formation from the amino groups of amino acids and from CO_2 by way of the arginine ornithine citrulline cycle of Krebs and Henseleit has been described in Chapter 14 The 24 hour urine of the adult will contain from 10 to 35 grams of urea, the amount being proportional to the protein intake Urea does not disappear from the urine on a protein free diet, since a minimal amount is produced under such circumstances by the catabolism of body proteins Analysis of the urine for urea has its chief value in tests of renal function, such as the urea clearance test, to be described in a later section Most analytical methods for urea involve the enzyme *urease*, obtained from vegetable sources such as jack bean meal This enzyme catalyzes the conversion of urea, by addition of one molecule of water, to ammonia and CO_2 The ammonia so formed can be measured by titration or by Nesslerization and colorimetry (9) A correction must be made for the ammonia already present in urine, or the ammonia may alternatively be removed by previous treatment with permutit Urea can also be measured by direct specific colorimetric methods which do not involve enzymes (1)

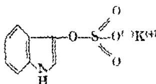
Urea and the other nitrogen containing solutes of the urine can be measured as a group by applying the Kjeldahl method for total nitrogen The

total nitrogen of a 24 hour specimen may run as low as 3 grams or higher than 20 grams of nitrogen, depending upon the protein intake Urines collected from subjects on the usual American diet run about 11 grams of nitrogen On a low protein diet, about 60 per cent of the total nitrogen is contributed by urea, on a high protein diet about 90 per cent The reason for this variation is the relative constancy of the other nitrogen containing solutes One such nitrogenous component is *ammonia* which is present in acid urines entirely as *ammonium salts* Under ordinary conditions ammonia contributes 0 2 to 0 8 grams of nitrogen to the total nitrogen of the 24 hour urine The ammonia content of urine is clinically significant in the evaluation of states of acidosis, and in making corrections of urea analyses when the urease method is used The aeration and permutit methods described by Folin (9) have not been superseded From 0 3 to 1 0 grams of *uric acid* is present in the usual 24 hour urine, contributing one third of its weight to the total nitrogen With unusually high purine intake in the diet, the uric acid may be as much as 2 grams Uric acid is the major end product of purine metabolism in man and in a very limited number of other mammalian species (see Chapter 7) On account of the *low solubility* of uric acid and urates, they often separate as precipitates from normal urines upon cooling Such precipitates are colored with urinary pigments—uric acid itself is colorless Uric acid will be considered in a later section as one of the components of stones of the urinary tract Urinary uric acid is also of significance in the diagnosis and follow up of cases of gout (see page 319) Analysis for uric acid is by colorimetric methods (9) *A total of* less than 100 mgm of allantoin and of purines other than uric acid is excreted per 24 hours The *creatinine* of urine, 1 to 2 grams per 24 hours contributes less than one gram to the total nitrogen The *metabolic origin* of creatinine has already been considered (see page 503) Analysis for creatinine is quite simple (9) but has little clinical significance Creatinine is the most constant of the urinary constituents in its rate of *output Creatine* is not a consistent urinary component, but is often found in the urines of normal women and children, and of patients with diseases involving muscular atrophy The excretion of amino acid nitrogen in the *urine* averages about 160 mgm per day Increased amino acid excretion is characteristic *of liver disease of* sufficient severity to impair deamination and urea formation, and also of the Fanconi syndrome (see page 510) Total amino acids can be measured most simply by a colorimetric method (9), the individual amino acids identified most *directly* by paper chromatography Amino acids in the urine may be free or conjugated *Histidine* is the free amino acid present in greatest amount The most *interesting of the conjugated amino* acids is *hippuric acid* (formula I) which is the conjugation product of glycine with benzoic acid *Its formation* in liver

and kidney and its excretion in the urine varies from 0 1 to 1 0 grams per day, depending upon the amount of benzoic acid and benzoic acid pre cursors (aromatic compounds with side chains containing an odd number of carbon atoms) in the diet Certain fruits such as cranberries, are rich in benzoic acid which is also used as a preservative for food products with a legal limitation of 0 1 per cent Hippuric acid is of historical interest as the first compound shown to be synthesized in the body This was demon strated by Wohler in a liver perfusion experiment The hippuric acid of the urine can be measured by direct precipitation, or by precipitation as benzoic acid after hydrolysis followed by weighing or titration The clinical value of such analyses is chiefly in connection with tests of liver function where measured amounts of benzoic acid are administered and their per



I Hippuric acid

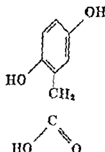


II Urinary indican (potassium salt of indoxylsulfuric acid)

centage recovery as hippuric acid in the urine is taken as an indication of the functional capacity of the liver This assumption is not strictly valid, since hippuric acid synthesis can also take place in kidney Urinary *indican* (formula II), an alkali salt of indoxyl sulfuric acid, appears in the urine in amounts up to 20 mgm daily From tryptophane, indol is formed by the action of intestinal bacteria The oxidation to indoxyl and the conjugation with sulfuric acid probably takes place in the liver In those days when 'intestinal autointoxication' was thought to be a major cause of human misery, the fluctuations in the indican content of urine were assiduously studied Several simple color tests for indican are described in texts of clinical pathology, but are seldom applied in current practice *Indol acetic* acid, a component of one of the urinary pigments is similar to indican in metabolic origin Several other urinary pigments, of known or unknown composition contain nitrogen, as do the small amounts of B vitamins and their derivatives excreted in the urine Finally we can close our listing of nitrogen containing urinary components with mention of the urinary

proteins which are normally less than 10 mgm per day and include certain *enzymes* The urine shows weak amylolytic, lipolytic and proteolytic activity the last *chiefly as uropepsin, which is identical with pepsinogen* The urinary lipase is probably of pancreatic origin the amylase of pancreatic and salivary origin Increase of urinary amylase may be of differential diagnostic value in cases of mumps and of pancreatitis

Most of the nitrogen containing urinary components mentioned in the preceding paragraph are derived from protein There are a few organic acids found in small amounts in the urine which are also protein derivatives but contain no nitrogen They include phenyl acetic phenyl propionic, p hydroxyphenyl lactic and p hydroxyphenylpyruvic acids All of these are quite normal and quite without clinical significance other than that they are excreted in increased amounts in scurvy (32) *Homogentisic acid*



III Homogentisic acid

(dihydroxyphenyl acetic acid) if present indicates a hereditary abnormality of tyrosine metabolism *alkaptonuria* This is a relatively harmless disease although sometimes accompanied by arthritis or pathological arterial changes (21) Homogentisic acid (formula III) has the properties of blackening by autoxidation in alkaline solutions and of reducing Benedict s and other alkaline copper reagents for sugars The urine of alkaptonurics turns black on standing exposed to air darkening from the top downward The formation of homogentisic acid from tyrosine is demonstrated by variations in output in alkaptonurics when tyrosine is fed or withheld The feeding of p hydroxyphenyl propionic *acid or of its alpha* hydroxy derivative does not increase the output of homogentisic acid whereas the alpha keto derivative will do so indicating that the last mentioned substance is an intermediate in the formation of homogentisic acid The normal oxidation of tyrosine ultimately involves breaking of the ring (see page 549) Small amounts of tyrosine normally escape ring breakage and are excreted as p hydroxyphenyl acetic and p hydroxyphenyl propionic acids In alkaptonuria the ring of tyrosine is not disrupted

Ochronosis, which is gray to brown or black pigmentation of sclera cartilage, or occasionally of the skin of the face, may occur with alkaptonuria or independently as a result of a similar failure of tyrosine metabolism The presence of *phenylpyruvic acid* in the urine is associated with imbecility or idiocy in a rare metabolic disease, phenylketonuria or phenylpyruvic oligophrenia, first observed by the Norwegian biochemist Folling in 1934 In such patients all of whom are mentally defective, about one gram of phenylpyruvic acid is excreted daily throughout their lifetimes The metabolic block in such patients has been shown to be their inability to hydroxylate phenylalanine to tyrosine (17)

In addition to aromatic organic acids *phenols*, present in amounts of the order of 300 mgm per 24 hours in urine are derived from aromatic amino acids Such formation of phenols from the aromatic rings of phenylalanine, tyrosine, and tryptophane is chiefly by bacterial deamination and decarboxylation in the intestine and to a lesser degree by similar reactions in the body tissues Certain phenols, indoxyl and skatoxyl contain nitrogen, while others such as phenol and *p* cresol do not The phenols are usually found in the urine conjugated with sulfuric acid, as in the case of indican

Oxalic acid and calcium oxalate Crystals of calcium oxalate dihydrate, identified under the microscope by their characteristic "envelope" appearance, are of very common and consistent occurrence in the urine sediments of healthy people Since calcium oxalate is extremely insoluble, such precipitation is to be expected from a solution containing both Ca^{++} and the oxalate ion Urine contains Ca^{++} in appreciable concentrations The amount of oxalate is much less, the range in normal urine being from 14 to 56 mgm per 24 hours (36) The source of the urinary oxalate is partly from oxalic acid and oxalates of the diet and partly from oxalate formed by metabolic processes within the body (15) Ingestion of spinach, which contains 0 8 to 0 9 per cent oxalic acid, is followed by increased oxalate output in the urine within 6 hours, except in achlorhydric patients, who showed such an increase only if hydrochloric acid was given with the spinach Some excretion of oxalate occurs, however, in subjects maintained on a diet low in oxalate, and in fasting subjects Calcium oxalate in the urine has an importance for students of medicine far out of proportion to the small amounts excreted Being solid, it can form stones (*calculi*) in the urinary tract, and even without calculus formation can be the cause of renal irritation with consequent bleeding, pain, or both The harmful effects of excessive urinary oxalate (*oxaluria*) briefly suggested here are explained more fully in a review by Jeghers and Murphy (16) together with a discussion of possible metabolic origins of oxalic acid in the body It appears that carbohydrate, rather than protein or fat, in the diet pro

motes the formation of oxalate The foodstuffs which directly contribut are certain vegetables beet tops, spinach, New Zealand spinach, sorre Swiss chard, and rhubarb stalks—all of these containing 0 5 per cent c more of oxalate expressed as oxalic acid Much of the oxalic acid is alread combined in the plant as insoluble calcium oxalate, hence these figure can be corrected downward as far as absorbable oxalate is concerned Th amount absorbed will also vary inversely with the amount of calcium pres ent in the other foods eaten at the same time The feeding of these high oxalate vegetables to unwilling infants and young children as a source o calcium is probably wasted effort, conversely, such vegetables, in reason able amounts, have been demonstrated to have no adverse effect on the calcium metabolism of healthy children or adults High oxalate vegetable should be excluded, as a reasonable precautionary measure from the diet of those who have had renal calculi or renal colic demonstrably the result of oxaluria Rhubarb stalks make a tasty pie filling, harmless in moderate amounts to healthy people, causing only a transient increase in the urinary content of calcium oxalate crystals *Rhubarb leaves contain toxic amounts of oxalic acid*, of the order of 1 2 per cent, and are too dangerous to be eaten, even in moderate amounts Fatal cases have been few, and have involved the eating of large amounts in one case (16) half a peck of the cooked leaves Nonfatal cases have been characterized by renal colic, blood in the urine, convulsions and sometimes gastrointestinal symptoms Others have eaten rhubarb leaves (amount not stated) with no subsequent dif ficulties Lest there be misunderstanding, it should be stated that only those vegetables specifically mentioned are of high oxalate content Beet roots contain only a little over 0 1 per cent of oxalate, and green vegetables such as celery, turnip leaves, endive kale, escarole asparagus, broccoli, cabbage Brussel sprouts, and cauliflower contain still less, as do other root vegetables, seeds and fruits Tea leaves are reported to contain about 0 2 per cent and powdered cocoa about 0 4 per cent oxalate calculated as oxalic acid

Other Organic Constitutents

Lipids occur only in minute concentrations in urine Normal urine con tains chemically detectable amounts of several of the steroid hormones and of metabolic products of steroids The epithelial cells and leukocytes of normal urine contain lipid material, as do all cells The fat droplets oc casionally seen in urine sediments are usually contaminants from dirty receptacles or from lubricants used in catheterization True *chyluria*, the presence of fat emulsified in the urine, occurs only when there is a fistula between the urinary bladder and the abdominal lymphatics This may occur as a result of malignancy or from infestation of the lymphatics with

the parasitic worm, *Filaria* Except after heavy intake of carbohydrates, sugars are not present in normal urine in amounts sufficient to reduce Benedict's solution The very small amounts of sugars present under fully normal circumstances can be measured by a modification of Folin's blood sugar method (9) *Glucuronic acid* in the form of conjugates with many drugs or with steroid hormones and their metabolism products occurs in the urine in a small and highly variable amount *Citric acid* a product of carbohydrate metabolism is excreted in the urine in amounts of 0 2 to 1 2 grams per day *Ascorbic acid* (vitamin C) is excreted in amounts which depend upon the intake and the subject's state of saturation (see page 703) The fat soluble vitamins are not urinary components Sugars and organic acids of urine are rapidly altered or destroyed by bacterial action if specimens are allowed to stand

Inorganic Constituents

Among the *anions* or acid radicals of the urine, Cl^- leads in quantity with a daily excretion in adults usually between 5 and 10 grams (140 to 280 mEq), varying almost exactly with the intake of Cl^- in the diet Diminished excretion as compared with intake occurs when Cl^- is lost through channels other that the urine, as by sweating, vomiting or diarrhea, or when fluid is being abnormally retained in the body as in the formation of edema or of large inflammatory exudates The chloride of the urine is usually determined by titration of excess silver nitrate with thiocyanate after precipitation of the Cl^- as AgCl (9) Although the output of chloride in the urine gives no direct diagnostic information, such measurements are often helpful in the assessement of the patient's state of fluid balance *Phosphate* ions leave the body both in the urine and by way of the feces The urinary output, expressed as P, is usually about one gram The output is so variable in its total amount and in its distribution between urinary and fecal channels that it is ordinarily impossible to decide whether phosphate output is normal diminished, or increased without placing the subject on a known diet and conducting a balance experiment The removal of the parathyroids leads to definite retention of phosphate, with diminished urinary output (see page 591), and conversely in hyperparathyroidism there is increased urinary excretion of phosphate as well as of calcium Positive phosphate balance occurs during growth and during pregnancy Measurement of urinary phosphate is usually carried out colorimetrically by the use of a molybdic acid reagent, which forms phosphomolybdic acid in acid solution Phosphomolybdate can be reduced to a blue molybdenum molybdate complex by several reducing agents which do not reduce molybdic acid itself (9) *Sulfur* is excreted in the urine in three groups of compounds—inorganic SO_4^{--}, conjugated sulfate (chiefly with phenolic com-

pounds), and neutral sulfur The last group mentioned includes compounds where the sulfur is present in forms other than the sulfate radical, examples of such compounds are cystine and taurine On a diet normal in protein content inorganic SO_4^{--} makes up about 90 per cent of the total sulfur excretion and the other two groups about 5 per cent each On a low protein intake the percentage of the total sulfur excreted as inorganic SO_4^{--} may fall to about 60 per cent, with neutral sulfur remaining about the same in absolute amount, and therefore constituting a greater portion of the percentage Since output of phenols is less on a low protein diet, the absolute amount of conjugated sulfate would also be less, but with the percentage possibly increased Inorganic sulfate can be measured gravimetrically by precipitation with $BaCl_2$ If the urine is previously submitted to acid hydrolysis, conjugated sulfates are released as SO_4^{--} and precipitation gives the sum of inorganic and previously conjugated SO_4^{--} If the urine is evaporated to dryness and ignited with a suitable oxidizing agent, then redissolved and precipitated with $BaCl_2$, the result measures the total sulfur, the sum of all three fractions On the basis of these three analyses, the fractions of urinary sulfur can be determined by subtraction The total sulfur of the urine ranges from 0 6 to 2 grams per day, expressed as S, and varying with the protein content of the diet In place of the tedious gravimetric methods, a precipitation with benzidine may be carried out on a semi micro scale (23) followed by titration Sulfate analyses in the urine have little clinical application *Bicarbonate* ion is present in urine, in concentrations increasing with the pH *Fluoride* is present in traces, varying with the intake (see page 594)

Of the urinary cations, Na^+ is quantitatively predominant, 3 to 5 grams (130 to 220 mEq) being excreted daily In Addison's disease (adrenocortical failure) the Na^+ output is excessive from failure of the renal tubules to reabsorb this ion Adrenal mineralocorticoids or synthetic desoxycorticosterone will correct this situation and in excess will bring about a similar loss of K^+ The output of K^+ is 1 to 3 grams (26 to 77 mEq) daily The urinary output of K^+ by the normal kidney continues even under fasting conditions, whereas Na^+ in the urine drops to very low levels in the absence of Na^+ intake Of the approximately one gram of Ca^{++} eliminated daily, from 10 to 40 per cent is usually eliminated in the urine, the remainder in the feces The daily urinary output of Mg^{++} ranges from 32 to 307 mgm with a mean of 103 mgm Some Mg^{++} is eliminated in the feces There is no iron in the fluid part of normal urine (19), but minute amounts are present in the cells of the urinary sediment *Lead* is not a physiological substance, but the environment of civilized life offers so many opportunities for ingestion of lead that it can usually be detected in the urine Amounts as high as 0 15 mgm per 24 hours have been observed in the urines of

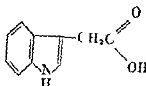
subjects who had no occupational exposure to lead and no symptoms of lead poisoning

URINARY PIGMENTS

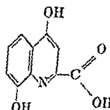
The pigments of normal urine have not been well or completely characterized *Uroerythrin* was proposed over a century ago as a name for the red pigment which colors deposits of uric acid and urates This pigment is red only in acid solution being yellow green in alkali It has absorption bands at 546–520 and 506–481 millimicrons *Urochrome* which is usually designated as the chief normal pigment of urine is not a single substance Descriptions of this pigment correspond to a mixture of pigments, containing among others uroerythrin and urobilin *Urorosein* was the name given to a red pigment obtained when certain pathological urines were strongly acidified and which was identified as an indolacetic acid derivative *Rosein* is the term proposed by Meiklejohn for a red pigment, not necessarily a single chemical entity developing in acid or acidified urine and extractable by amyl alcohol but not by chloroform or toluene, and decolorized by light and by alkali Oxidation of indole 3 acetic acid (formula IV) produces a rosein like pigment with a weak absorption band at 500 plus a stronger band at 530 millimicrons comparable to the absorption bands of uroerythrin and urorosein Indolacetic acid has been identified in urine, chiefly for the reason that it is a plant hormone a hetero auxin promoting epinasty the downward curvature of leaves by extra growth on the upper side as well as root growth Rosein and hetero auxin output in the urine are increased simultaneously by liver disease and by nutritional deficiencies A *roseinogen* has been extracted by Meiklejohn from the urine of a patient with cirrhosis The reactions of the roseinogen are those of indol 3 acetic acid except that the roseinogen is more soluble It has been suggested that the increased solubility is achieved by conjugation with glycine Roseinogen has not been crystallized *Urobilin* and *urobilinogen* are bile pigment derivatives and occur normally Urobilin has a brown color, urobilinogen is colorless Urobilinogen responds to the familiar Ehrlich aldehyde reagent which is paradimethylaminobenzaldehyde in hydrochloric acid Both urobilin and urobilinogen have fluorescent zinc salts which can be used as a further means of identification Chief clinical interest in these substances lies in their absence in obstructive jaundice, in which situation no bile pigment derivatives can be absorbed from the small intestine since no bile pigment is there Absence of these two substances from the urine, *then is an indication of obstructive jaundice In their* place we find unchanged bile pigment particularly bilirubin In clinical laboratories this is usually identified by the familiar shaking and foam test which is actually more dependable than most proposed chemical tests for bile pigments

Of the chemical tests, most satisfactory is Harrison's which uses pap strips saturated with barium chloride and dried The paper specified S & S No 470 These papers are dipped into the urine for ten seconds ar a drop of Fouchet's reagent at the surface line gives a green color if bilirub is present Fouchet's reagent is 25 per cent trichloracetic acid containin 0 9 per cent ferric chloride A quantitative modification of this test utilizin spectrophotometric measurement of the color has been described (35

Melanin is a rare abnormal pigment sometimes found in the urine of pa tients with the highly malignant melanotic type of tumors Melanin n itself is black It is often excreted in the form of a colorless precursor whicl darkens on exposure to the air Since the harmless homogentisic acid be haves in the same way differentiation here is important Direct identifi cation of homogentisic acid can be easily done by utilizing the fact that it darkens ordinary photographic paper in strong alkaline solution in full daylight Techniques for the concentration and identification of melanin



IV Indole-3 acetic acid



V Xanthurenic acid

have been summarized by Rothman (33) The pigment isolated from melanotic urine has properties similar to the pigment obtained from human red hair

Xanthurenic acid (formula V) is a yellowish substance a product of the abnormal metabolism of tryptophane in pyridoxine-deficient subjects or experimental animals

Porphyrins Normal urine contains a small amount 0 1 mgm per 24 hours or less of coproporphyrin and a very much smaller amount of uro porphyrin (27) Excess porphyrin may be the result of a primary disease porphyria or may occur as a symptom of numerous disorders including chronic alcoholism liver or hemolytic disease poisoning by quinine cincho phen various hypnotics sulfonamide drugs, and various inorganic posons particularly lead

De Langen and ten Berg (7) found that increased excretion of copro porphyrin in the urine was an early sign of lead poisoning In such cases porphyrin is not excreted in amounts sufficient to produce a visible change in the color of the urine As a semi quantitative screening test a few drops of glacial acetic acid and 2 ml of ether are added to 20 ml of the suspected

urine The mixture is shaken and examined under ultraviolet light, in which the ether layer fluoresces With urines of normal porphyrin content the fluorescence is light blue to green but gradually deepening red fluorescence appears as the porphyrin content increases In case of an equivocal result a quantitative method can be employed which is also described in the paper cited Increased urinary excretion of coproporphyrin is also characteristic of regurgitation jaundice (see page 464) resulting either from biliary tract obstruction or from liver damage There have also been a few reported cases of individuals who excrete large amounts (1 to 6 mgm per day) of coproporphyrin without demonstrable cause other than a hypothetical metabolic error These patients show no consistent symptoms associated with their coproporphyrinuria In all these instances of excess coproporphyrin excretion the porphyrin is the type III isomer

Patients with *congenital porphyria* excrete a different porphyrin uroporphyrin Type I along with coproporphyrin I often show photosensitivity of the skin and red fluorescence of teeth and bone This is a rare disease and most of the diagnosed cases have been written up Much of our knowledge of this disease comes from the careful study of a single patient Petry Petry died (aged 32) on January 21 1925 and was autopsied the same day by Borst His organs were analyzed for porphyrins and other pigments by Konigsdorffer Their combined report (3) gives full details of the histological and histochemical (fluorescence microscopical) findings with a chapter based upon their findings discussing congenital porphyria as a *Konstitutionsanomalie* Deficient formation of hemoglobin myoglobin and cytochromes could not be demonstrated nor could deficient mechanisms for destruction of blood pigments be shown nor could they prove disturbance in intermediary bile pigment metabolism nor show that the abnormal porphyrins were by products of bile pigment metabolism They conceived the fundamental error in congenital porphyria to be the persistance of an early phylogenetic and ontogenetic developmental stage in which the independent synthesis of porphyrin by erythroblasts occurs Erythroblasts are produced in excess and are also destroyed at an excessive rate with liberation of the porphyrin Uptake of dietary porphyrins or of porphyrins formed by intestinal bacterial synthesis or bacterial degradation of chlorophyll was considered but could not be demonstrated

Acute porphyria with abdominal neurological and psychiatric symptoms, may appear at any age This disease is not definitely congenital although there appears to be an inherited predisposition evidenced by familial incidence Both causation and therapy in this disease are still obscure Uroporphyrin and coproporphyrin mainly Type I are excreted in varying and sometimes very large amounts (30 mgm per day) accompanied by *porphobilinogen* a substance which reacts strongly with

Ehrlich s aldehyde reagent and also accompanied by *the Waldenstrom porphyrin* which is uroporphyrin I combined with an unidentified Type III *porphyrin Of all* the porphyrias this is the most serious sometimes causing death at the first attack in other instances leaving the patient in a paralyzed state Procedures for the identification of the several urinary porphyrins are *summarized by Ham (13)*

ABNORMAL URINARY CONSTITUENTS

The presence of *sugars* above the limit *of the normal concentrations and* of the ketone bodies (see page 523) in amounts greater than 20 mgm per day *in the* urine is abnormal *The* implications of these substances as ab normal urinary components have already been discussed Normal urine contains very little protein up to 12 mgm per 100 ml with the mode *between 0 and 2 mgm (12) Larger amounts of protein are abnormal Coagulable protein* (usually called *albumin* and actually mostly albumin with some admixture f serum globulin) can be detected by the famil ar heat and acetic acid test in any amount greater than normal Technical note the urine should be acidic for the preliminary heating but not ex cessively acidified since *soluble acid* metaprotein may be forme l *Sulfo* salicylic acid usually applied in urine examination as a ring test is a popular but quite expensive protein precipitant Nitric acid is traditional for this *purpose and is notably cheaper* but *has certain well recognized* d sad vantages—the staining of the analyst s skin and possible damage to clothing and woodwork the precipitation of urea nitrate or uric acid or both from concentrated urines (dilute and try again) the precipitation of *resin acids* (components of numerous pharmaceutical preparations cough syrups in particular) *and the formation by oxidation of a dark pigment band which* obscures the albumin ring Robert s reagent (one volume of concentrated nitric acid plus 5 volumes saturated aqueous magnesium sulfate solution) eliminates the last mentioned difficulty and is equally sensitive to albumin as nitric acid If large numbers of urines are to be tested for albumin a very satisfactory timesaving *device is to test all* specimens by *the ring* test using Robert s reagent then recheck the positives with the heat and acetic acid test to eliminate the false positives

Albuminuria The presence of albumin i e heat coagulable protein in the urine is always abnormal but is not diagnostic of any particular disease and indeed may occur *in a healthy person under conditions of* physical stress Evidence gained from animal studies shows that the glo merular filtrate contains small am[illegible] lasma protei is which indicate *that the glomerulus is not a per*[illegible] r The renal tubule cells nor mally reabsorl[illegible] in ma[illegible] ine practically protein free

Albuminuria may therefore arise from increased protein leakage through damaged glomeruli, or from failure of protein reabsorption by non functional tubule cells, or both There is no evidence that tubule cells extrude protein into the urine The highest outputs of urinary albumin are seen in those diseases where tubules are known to be affected

Albuminuria may occur in the following situations

(1) From stress This is usually a physical stress (emotional stress is more likely to cause glycosuria), such as a marathon run, or long immersion in cold water

(2) From postural defect Some healthy people, usually adolescents or young adults have albuminuria when up and about and no albuminuria when resting in a horizontal position This appears to be the result of a change in kidney circulation, and has in some cases been associated with lumbar lordosis, or with a movable kidney Such albuminuria is often associated with the late growth period and may disappear completely with the attainment of full growth Before pinning a diagnosis of Bright's disease upon any youngster on the basis of albuminuria the physician should make the simple check of testing a urine specimen collected over a time period while the patient is resting quietly in bed

(3) From pre-renal disease Circulatory diseases, and occasionally fevers, may cause local passive congestion or other circulatory disturbance in the kidneys leading to albuminuria

(4) From renal disease This is of course the most common cause of albuminuria, and the disease may be practically any to which the kidney is subject, including congenital malformations, nephritis, nephrosis, tuberculosis, tumors, infarcts, and poisonings The heaviest albuminurias occur in nephrosis and in amyloid disease, with as much as 5 per cent coagulable protein in the urine With most other lesions less than one per cent albumin is found

(5) From post renal sources Admixture of blood, pus, sperm, or other albuminous material from the lower genito-urinary tract gives rise to albuminuria

Mucoprotein is not heat coagulable, and is not strictly an abnormal substance in the urine It is secreted by the cells of the urinary tract, and is normally present in small amounts In any irritation or infection of the urinary tract it is greatly increased in amount and is often visible grossly as shreds or microscopically as threads

Proteoses of the urine are a rather doubtful and clinically unimportant category of proteins They are not heat coagulable, but will precipitate with nitric acid, or by saturation with ammonium sulfate after removing heat coagulated albumin by filtration Proteoses have been reported in

the urine in almost every type of disease, hence they have no diagnostic significance Some contribution of protease to urine may occur from prostatic secretions

Bence-Jones protein On November 1 1845 Henry Bence-Jones physician to St George s Hospital, examined urine specimens from a 47 year old grocer who had been out of health for thirteen months The urine if heated and left to cool became solid This solid redissolved by heat and again formed on cooling On January 2 of the next year the patient died "The following day I saw that the bony structure of the ribs was cut with the greatest ease and that the bodies of the vertebrae were capable of being sliced off with the knife This patient was a case of what we now call *multiple myeloma* (2) and the curiously behaving solid we now call the Bence-Jones protein In a urine slightly acidified with acetic acid the Bence-Jones protein shows its presence by precipitation at temperatures between 45 and 60°C and redissolves at 100°C If albumin is also present it may be filtered off at 100°C and the urine upon cooling will again throw down the Bence-Jones protein at about 60°C The Bence Jones protein is found in the urine of a little over half of the pathologically proven cases of multiple myeloma which is a neoplastic disease characteristically involving the red marrow bones—sternum ribs and vertebrae The tumor cells resemble plasma cells The x ray appearance is of multiple rounded defects The disease is usually rapidly fatal The Bence Jones protein may be eliminated in the urine in very large quantities up to 70 grams in a day It is considered by most authorities to be a tumor cell modification of serum globulin Immunological evidence leads to the conclusion that it is related to human proteins but this conclusion has been challenged by Dent and Rose (8), who make the rather daring suggestion that multiple myeloma is a virus disease and the Bence-Jones protein a constituent of the virus The Bence-Jones protein is not specific for multiple myeloma It has been reported in many other bone diseases also in myxedema nephritis and chronic leukemia (there is however, a leukemic type of multiple myeloma) These occurrences without association with multiple myeloma are rare and some are doubtful

Blood In speaking of blood in the urine we distinguish *hematuria* as the presence of red blood discs in the urine as the result of hemorrhage into the urinary tract The observation of hematuria is made or confirmed with the microscope As opposed to hematuria *hemoglobinuria* is the presence of free dissolved hemoglobin in the urine This is evidence of hemolysis occurring in the blood vascular system There are numerous causes of such hemolysis including transfusions of incompatible blood severe infections with hemolytic micro-organisms such as the hemolytic streptococcus malaria typhoid and yellow fever Hemolysis may occur with severe

burns or chilling, congenital or acquired hemolytic jaundice and numerous types of poisoning e g, fava beans Some few individuals develop hemoglobinuria after exercise (10) The color of urine is not a reliable guide to the presence of hemoglobin Ordinarily dependence is placed on one or another of the modifications of the peroxidase reaction The urine normally contains no peroxidase True enzymatic peroxidase is present in white cells The hemoglobin of red cells has a peroxidase action In the presence of hydrogen peroxide, hemoglobin, acting like a peroxidase, catalyzes the oxidation of a number of organic substances The one commonly used in testing for blood is benzidine less commonly guaiac or o tolidine, all of which yield blue oxidation products Since leukocytes contain peroxidase, the presence of large amounts of pus in the urine may give a false positive reaction for hemoglobin This reaction of leukocyte peroxidase will be absent if the urine is boiled, whereas the pseudoperoxidase action of hemoglobin is not affected The easiest differentiation is by microscopic identification of the leukocytes Compounds closely related to hemoglobin may also occur, such as methemoglobin, rarely from a congenital metabolic fault, more commonly from oxidizing agents such as nitrites or chlorates used as drugs, and also myoglobin, the red respiratory pigment of muscle which is found in the urine after crush injuries These pigments give a peroxidase reaction similar to that of hemoglobin They can be distinguished best by the use of the spectroscope

UROLITHIASIS

The formation of stones in the urinary tract occurs by the precipitation, usually in crystalline form, of urinary solutes, together with the aggregation of the precipitated material into a compact mass The substances found in urinary concretions are relatively few in number, and most of them have been recognized as stone formers for many years Table 43 shows the substances found in 1000 calculi examined by Prien (29) Certain of these warrant more detailed description

Calcium oxalate has already been discussed as a normal urinary component The crystals observed in many normal urine specimens are calcium oxalate *dihydrate*, which does occur in calculi The *monohydrate* is a more common stone component, as shown by the table Calcium oxalate calculi are characteristically found in patients with normal acid urines, and are not associated with any other disease or abnormality than oxaluria

Calcium oxalate monohydrate stones are usually of 'hempseed", 'mulberry', or 'jackstone' shape, and with a smooth brown surface Stones containing the dihydrate (pure or mixed) are more likely to be rough, often with projecting crystalline spicules, which add to the trauma produced by the stone in traversing the narrower portions of the urinary tract Such

stones are often stained with blood pigment Calcium oxalate can be crudely and simply identified as a stone component by the fact that after ignition (to dull red heat until all visible carbon is burned away) of a small bit of the powdered stone it will effervesce with a drop of dilute HCl, and will

TABLE 43

Substances found in 1000 urinary calculi

Pure calcium oxalate			
Calcium oxalate monohydrate	137		
Calcium oxalate dihydrate	4		
Calcium oxalate (mixed)	186	327 =	32 7%
Calcium oxalate plus apatite			
Calcium oxalate monohydrate plus apatite	72		
Calcium oxalate dihydrate plus apatite	47		
Calcium oxalate (mixed plus apatite)	224	343 =	34 3%
Apatite (pure)	34	34 =	3 4%
$MgNH_4PO_4 \cdot 6H_2O$ (pure)	3	3 =	3%
$MgNH_4PO_4 \cdot 6H_2O$ plus apatite	155	155 =	15 5%
$MgNH_4PO_4 \cdot 6H_2O$ plus apatite plus calcium oxalate (mixed)	32	32 =	3 2%
Calcium hydrogen phosphate dihydrate			
Pure	2		
Mixed	17	19 =	1 9%
Uric acid			
Pure	47		
Mixed	11	58 =	5 8%
Cystine			
Pure	22		
Mixed	7	29 =	2 9%
		1000 =	100 0%
Sodium acid urate			
Microscopic amount in one mixed uric acid calculus			

not do so prior to ignition The heating converts the oxalate to carbonate This observation will not of course differentiate the two hydrates For exact crystallographic methods of identification of calcium oxalate and of other stone components, the paper of Prien and Frondel (30) should be consulted

Apatite stones, with composition similar to that of bone salt (see Chapter 15), together with the other *phosphatic* calculi, are associated in a general way with alkaline, infected urines, although this association is by no means uniform The alkaline earth phosphates as a group are less soluble as pH increases Infection of the urinary tract, particularly with organisms which convert urea into ammonium carbonate, often produces an alkaline urine and causes the precipitation of apatite and other phosphatic deposits Excess excretion of phosphate, as in hyperparathyroidism (see page 591), may also lead to the production of such calculi, even in acid urine and in the absence of infection A consistently alkaline ash diet or the frequent use of sodium bicarbonate may result in an alkaline urine and formation of phosphatic calculi Such calculi have been found in cattle also stones containing calcium carbonate which is not found in human uroliths Human phosphatic calculi may form rapidly and grow to such size as to fill the renal pelvis and calyces ("staghorn" calculi) Immobilization or long confinement in bed increases the probability of formation of phosphatic calculi (6)

Uric acid will be noted as one of the less common stone formers The stones are usually small and colored with urine pigment There is no significant association of the formation of uric acid calculi with diet or with any other disease, with the exception of gout (see page 319) and here the association is not striking The kidneys of newborn babies often contain heavy deposits of urates the so called "uric acid infarcts", which soon disappear Their presence is not considered pathological *Xanthine* calculi did not appear in Prien's series, but have been occasionally reported (3)

Cystine calculi are smooth relatively soft may reach considerable size, particularly in the bladder, and are present only in cases of *cystinuria* This is inborn error of metabolism in which cystine can not be metabolized for energic purposes by the body, although it is still utilized in protein structure (see Chapter 2) Since cystine is the least soluble of the naturally occurring amino acids, precipitation frequently occurs, crystals of cystine can usually be found in the urine of cystinurics, and almost inevitably a calculus will form Such calculi are usually large and single and are commonly found in the bladder Crude identification is easy by the "burnt feathers" odor on ignition Renal concretions of indigo cholesterol, and the fatty "urostealiths" are pathological curiosities which remain inadequately explained

THE MEASUREMENT OF RENAL FUNCTION

A very large number of kidney function tests have been used by clinicians and physiologists Most of these tests fall into one of three classes

(1) Those which are based upon the excretion of normal urinary components,

(2) Those which are based upon the excretion of foreign substances, and

(3) Those which are based upon the study of a single component, either normal or foreign, simultaneously in the blood and in the urine All of these test the excretory function of the kidney The regulatory function of the kidney is demonstrated by the maintenance of normal levels of significant substances, e g , urea, uric acid, or inorganic phosphate, in the blood, and by the maintenance of a normal acid base balance Analysis for blood components is not in itself usually designated as a renal function test, but is a valuable diagnostic aid

Concentration and dilution tests are the chief representatives of the first category of tests, where normal urinary constituents are measured Usually, instead of selecting a single component, measurement is made of the specific gravity, which is a rough index of all components One procedure commonly used combines a concentration and a dilution test the patient remains on his usual diet and collects 2 hour specimens punctually during the waking day, and collects the night urine ' as a single 12-hour sample At least a pint of fluid is taken with each of the three meals, but no solid food between meals, and no food or fluids during the 12 hour 'night' period This procedure contrasts a day with ample fluid with a night of no fluid In the patient with adequate powers of urinary concentration and dilution the variation in specific gravity between the highest and the lowest in the several specimens should be at least 0 009, the night urine should have a specific gravity of at least 1 018 and a volume not over 500 ml For a description of pure concentration and pure dilution tests consult Ham (13) Hayman and his collaborators (14) plotted the number of glomeruli counted post mortem against the maximal specific gravity of the urine measured before death The value for the maximal specific gravity fell consistently with the number of glomeruli until the latter figure reached 750,000 per kidney At lower values of the glomerulus count the specific gravity remained fixed at 1 010 The normal kidneys contain about a million glomeruli each

Phenolsulfonephthalein (PSP or phenol red) offers many advantages as a foreign substance useful in renal function testing The substance keeps indefinitely in solution, can be sterilized by boiling causes no pain or local reaction upon injection, and is excreted entirely through the kidneys Although rate of urine volume output does not greatly affect the rate of

excretion of PSP, except with severe oliguria it is best to provide adequate urine volumes during the test by giving the patient 600 ml of water The usual dose is 6 mgm of PSP dissolved in one ml saline If given by subcutaneous or intramuscular injection a latent period or appearance time' of 3 to 10 minutes elapses before the dyestuff appears in the urine Its presence can be detected by alkalizing the urine with NaOH, which turns the dye from its acid yellow color to its alkaline red color Quantitative estimation of the amount excreted can be made by comparison with known color standards Normally from 40 to 60 per cent of the injected dose is eliminated in the first 70 minutes after subcutaneous or intramuscular injection and from 60 to 85 per cent in the total test period of 130 minutes Total eliminations less than 25 per cent for the test period indicate serious loss of excretory ability After intravenous injection there is no latent period, and from 28 to 51 per cent of the dose is eliminated within 15 minutes in patients with normal renal function In cases where unilateral renal disease is suspected the outputs of the two kidneys can be studied separately by the use of ureteral catheters The PSP test has certain limitations In obese individuals, in cases of hypothyroidism, and in situations where circulation is slow, the PSP output is diminished, suggesting renal damage which may not exist The PSP test is not sensitive to early or minimal changes in renal disease where fixation of specific gravity and clearance tests (see next paragraph) may already be informative During acute diseases, such as influenza the PSP output may be temporarily decreased with a return to normal with recovery

Clearance tests are the most widely used methods involving simultaneous measurement of blood concentration and urine output of a given substance Such tests measure the excretory function of the kidney, usually disregarding the regulatory function The simplifying assumption is made that the action of the kidney is to clear' the blood of a certain substance Clearance is defined as the *calculated volume* of plasma cleared of a given substance in one minute Actually plasma is not usually cleared of any substance by passage through the kidney Clearance is spoken of most accurately as a virtual volume or more bluntly as a fictitious volume

The general formulation of clearance involves no mathematics other than simple arithmetic Clearance, in ml per minute, is

$$\frac{UV}{P}$$

where U and P represent respectively the concentrations of the given substance in urine and in plasma, measured in the same units, and V is the volume of urine per minute *Glucose* normally has a clearance of zero, since it is completely reabsorbed by the renal tubule cells, giving U a value of zero

Urea clearance was the original application of the clearance principle and is the test most commonly used clinically. Urea is only partially returned to the plasma by the renal tubule cells. The assumption that simple diffusion of urea occurs from glomerular filtrate into tubule cell fits most data better than the assumption of active reabsorption of urea by the cell, although there is not complete agreement on this point (4). At any rate a portion of the urea is excreted; hence urea clearance can be calculated. With copious water excretion the amount of urea excreted in unit time is independent of the volume output. Urea clearance under these conditions is called *maximum clearance* and is calculated by the simple formula already stated. The normal maximum clearance of urea is 60 to 100 ml., with a mean of 75. At rates of water excretion less than 2 ml. per minute the urea output has been observed to increase in direct proportion to the square root of the urine volume. In this event the square root of V is used in place of V in the formula, giving the *standard clearance*, defined as the "volume of blood which one ... of urine excreted in 1 minute suffices to clear of urea" (25). The value of standard clearance ranges from 41 to 65 ml. normally, with a mean at 54. Unlike the maximum clearance, the standard clearance has no direct physiological significance but is an empirical expedient for clinical purposes only. Note that both clearances can not be calculated from the same data. In calculating urea and other clearances it is advisable in persons of unusual body configuration to multiply V by a correction for surface area:

$$\frac{1.73}{\text{actual surface area of patient}}$$

The value of 1.73 is the average adult surface area in square meters. The surface area of the patient can be obtained by the use of prepared weight-height-area tables. Clearance as well as blood volume and kidney weight has been found to parallel surface area more closely than height, weight, or any other accessible physical measurement.

Inulin clearance is considered to be the result of glomerular filtration with no reabsorption; therefore the maximum clearance of inulin represents the *filtration rate*, which in men has a mean value of 127 ml. of glomerular filtrate produced per minutes, in women 117 ml. These figures represent the upper limit of clearances by glomerular filtration alone. If the clearance of any substance is greater than the simultaneously measured inulin clearance, it is concluded that that substance is excreted by the tubules. *Thiosulfate* has an identical clearance with inulin. Neither inulin nor thiosulfate will leave extracellular fluid to enter cells.

Creatinine clearance at normal blood levels is identical with that of inulin. After ingestion of creatinine, the clearance may increase to a maxi

mum of 175, indicating that tubular excretion is taking place in addition to glomerular filtration

Diodrast clearance, at relatively low diodrast (3,5-di iodo-4 pyridone N acetic acid) concentrations in the blood is the result of glomerular filtration plus maximal tubular excretion, and may reach a value of 740 ml, which represents the physiologically functional blood flow through the kidney per minute The 740 ml of plasma represents 1200 ml of whole blood, about one fourth of the cardiac output per minute Of all the substances so far mentioned diodrast alone is actually "cleared" from the plasma by the kidney Another such substance is *p aminohippuric acid* (PAH) which has a clearance identical with that of diodrast

The maximal rate of tubular reabsorption of glucose, or glucose Tm, is observed at concentrations of glucose in the arterial plasma above a saturation value which in men is approximately 0 3 per cent and in women, 0 26 per cent Note that this value is considerably higher than the glucose threshold, above which glycosuria occurs The threshold is quite variable in different individuals and somewhat variable in the same individual In 80 per cent of normal subjects it falls within the range of 0 14 to 0 19 per cent The glucose threshold is usually measured in venous blood, but this does not account for the great difference between the threshold and the saturation value for glucose absorption According to Smith (34) certain nephrons with high glomerular activity become saturated at lower levels of plasma glucose therefore permitting escape of glucose into the urine Glucose Tm is calculated by multiplying the saturation value by the filtration rate or inulin clearance It was 375 ± 79 7 mgm glucose per minute in 24 normal men studied by Smith and his colleagues, and 303 ± 55 3 mgm in 11 normal women In patients with renal disease, glucose Tm is proportional to the number of intact nephrons, since both glomeruli and tubules are involved

The maximal diodrast or PAH excretion (diodrast Tm or PAH Tm) is a measure of tubular function alone, since the part played by glomerular filtration is constant and negligibly small The diodrast Tm or PAH Tm minus the glucose Tm measures the number of aglomerular tubules Since inulin excretion is purely glomerular, inulin clearance minus glucose Tm measures the number of glomeruli with functionless tubules For further consideration of renal clearances and the physiological information which they yield, consult the monograph by Smith (34)

The generation of high-energy phosphate bonds, which normally accompanies the aerobic oxidations catalyzed by the cyclophorase system (see page 199), is inhibited by 2,4-dinitrophenol This same drug greatly reduces the renal tubular excretion of *p* aminohippuric acid, of diodrast, and of phenol red, with no significant change in renal hemodynamics (26)

These results in dogs suggest that phosphate bond energy is made use of in tubular excretion

The concept of clearance has been applied to excretion by channels other than the kidney (20) If the small intestine is perfused with 2 per cent sodium sulfate at a rate between 23 and 30 ml per minute, the concentration of urea in the outflow is about equal to the urea concentration of the patient s plasma The urea clearance is therefore about equal to the ml of perfusing fluid per minute

The *artificial kidney* is a device by which blood is submitted to dialysis outside the body and then returned to the circulation The solution against which the blood is dialyzed contains glucose and the significant ions of the blood plasma Such an artificial kidney (24) can remove as much as 10 grams of urea nitrogen per hour from the patient's blood

RENIN AND EXPERIMENTAL HYPERTENSION

If the blood supply to one kidney of an experimental animal is decreased an increase in blood pressure occurs which returns to the preoperative level within 6 weeks Such decrease of blood supply was first accomplished by Goldblatt, using a clamp which constricted but did not close the renal artery A similar result can be achieved by enclosure of the kidney within a tight covering or by inducing injury with resulting scar tissue contraction

If an animal is made hypertensive by the above procedure, and the un operated normal kidney is removed or if both kidneys are rendered is chaemic, the hypertension is permanent In dogs with bilateral ischaemic kidneys such hypertension may persist as long as 6 years, with no loss of renal excretory function as measured by the usual functional tests

Houssay and his co workers demonstrated, by such procedures as trans planting an ischaemic kidney to the neck obtaining venous blood directly from such a kidney and injecting the blood into another animal, that the hypertension in animals with ischaemic kidneys resulted from the presence of a pressor substance in the blood The pressor substance is the product of the reaction of a renal enzyme *renin* upon its specific substrate, *hyper tensinogen* which is one of the plasma globulins The product of this enzymatic action is a polypeptide designated as *hypertensin* or *angiotonin* The production and storage of renin seems to be limited to the renal cortex A reduction in pulse pressure, with the substitution of a steady for a pulsate blood flow, seems to be the major physiological factor in producing or liberating renin The actual pressor substance the polypeptide, under goes destruction by further enzymatic activity The concentration of pressor substance in the blood depends upon the balance between the two enzyma tic processes by which it is formed and destroyed In order to detect hypertensin in blood, it is necessary to check the action of the destructive

enzymes by immediately cooling the blood to 0°C (11) The enzymes which destroy angiotonin most effectively are those obtained from kidney extract, which contains many unidentified enzymes Destruction is accelerated by the addition of oxidized cytochrome both *in vitro* and *in vivo*, as evidenced by reduction of pressure of normal rats Diminished response to repeated doses, or *tachyphylaxis* of renin is explained as the result of using up the available hypertensinogen The origin of hypertensinogen has been located in the liver, since the substance can not be demonstrated after hepatectomy, or when the liver is severely damaged by carbon tetrachloride poisoning

The degree to which renin participates in the regulation of human blood pressure and in the causation of human hypertension has not been established In a limited number of human cases, unilateral renal disease has been accompanied by hypertension which was relieved by the removal of the diseased kidney, when the contralateral kidney was normal It has not been possible to detect renin or angiotonin in cases of chronic hypertension in the human

REFERENCES

1 ARCHIBALD R M Colorimetric determination of urea J Biol Chem 157. 507–518 1945

2 BAIRD, I D AND HECK I J Multiple myeloma A review of eighty three proved cases J. A M A 133 147 157, 1947

3 BERMAN, I S Twenty second case of xanthine urinary calculus J Urol 61 420, 1948

4 BIERING T Renal excretion of urea Acta med scandinav, 136 Suppl 234 33–40, 1949

5 BORST, M, AND KOENIGSDOERFFER H *Untersuchungen uber Porphyrie mit besonderer Berucksichtigung der Porphyria Congenita* Leipzig, S Hirzel, 1929

6 BOYD, M L The formation of renal calculi in bedridden patients J A M A 116 2215–2247 1941

7 DE LANGEN, C D, AND TEN BERG J A G Porphyrin in the urine as a first symptom of lead poisoning Acta med scandinav 130 37–44, 1948

8 DENT, C E, AND ROSE, G A The Bence Jones protein of multiple myelomatosis its methionine content and its possible significance in relation to the etiology of the disease Biochem J 44 610–618 1949

9 FOLIN O *Laboratory Manual of Biological Chemistry* 5th Ed New York, Appleton Century 1934

10 GILLIGAN D R AND BLUMGART H L March hemoglobinuria Medicine 20 341 395 1941

11 COLLAN F *et al* Hypertensin in systemic blood of animals with experimental renal hypertension J Exper Med 88 389–400 1948

12 GUNTON R AND BURTON A C On the concentration of protein in samples of normal urine measured by its surface activity J Clin Investigation 26 892–898 1947

13 HAM T H A *Syllabus of Laboratory Examinations in Clinical Diagnosis* Cambridge Mass, Harvard University Press 1950

14 HAYMAN J M JR *et al* Renal function and the number of glomeruli in the human kidney Arch Int Med 64 69–83 1939

15 HERKEL W AND KOCH K Untersuchungen zur Oxalsäure Ausscheidung insbesondere bei Nierensteinkranken Deutsches Arch klin Med, 178 511–537, 1936

16 JEGHERS H AND MURPHY R Practical aspects of oxalate metabolism New England J Med 233 208–215 238–246 1945

17 JERVIS G A Phenylpyruvic oligophrenia The position of the metabolic error J Biol Chem 169 651–656 1947

18 KEYSER L D *et al* Studies on the solution of urinary calculi experimental and clinical aspects J Urol 59 826–841 1948

19 LINTZEL W Zur Frage des Eisenstoffwechsels IV Ueber das Harneisen Ztschr Biol 87 157 166 1928

20 MALUF N S R Urea clearance by perfusion of the intact small intestine in man J Urol, 60 307 315 1948

21 MAUSER C I Alkaptonuria J A M A 146 815–816 1951

22 McCANCE R A Renal function in early life Physiol Rev 28 331–348 1948

23 McKITTRICK D S AND SCHMIDT C L A Determination of sulfate by the benzidine method Arch Biochem 6 411–417, 1945

24 MERRILL J P *et al* The use of an artificial kidney J Clin Investigation 29 412–424 1950

25 MÖLLER E *et al* Studies of urea excretion II The relationship between urine volume and the rate of urea excretion by normal adults J Clin Investigation 6 427–465 1928

26 MUDGE G H AND TAGGART J V Effect of 2 4 dinitrophenol on renal transport mechanisms in the dog Am J Physiol 161 173–180, 1950

27 NICHOLAS, R E H AND RIMINGTON C Qualitative analysis of the porphyrins by partition chromatography Scandinav J Clin & Lab Invest 1 2 11 1949

28 PITTS R F Acid base regulation by the kidneys Am J Med 9 356–372, 1950

29 PRIEN E L Studies in urolithiasis II Relationships between pathogenesis structure and composition of calculi J Urol 61 821–836 1949

30 PRIEN E L AND FRONDEL C Studies in urolithiasis I The composition of urinary calculi J Urol 57 949–991 1947

31 RANDALL A An hypothesis for the origin of renal calculus New England J Med 214 234 242 1936

32 ROGERS W F AND GARDNER F H Tyrosine metabolism in human scurvy J Lab & Clin Med 34 1491–1501 1949

33 ROTHMAN S Studies on melanuria J Lab & Clin Med 27 687–692 1942

34 SMITH H W *The Kidney Structure and Function in Health and Disease* New York Oxford University Press 1951

35 THOMAS G F AND KITZBERGER D M Spectrophotometric method for determination of urinary bilirubin J Lab & Clin Med 22 1159–1192 1948

36 WIDMARK, E M P Oxalic acid in the urine Acta med scandinav, Suppl 26 340 1928

PART V

Pathology

Increased requirement for a vitamin or a group of vitamins may be th result of growth pregnancy, lactation or physical exertion or may b secondary to diseases such as fever or hyperthyroidism which accelerat metabolic processes The need for certain vitamins increases with increase carbohydrate intake hence patients who are being nourished chiefly by intravenous injections of glucose are commonly given thiamine and othe water soluble vitamins in proportion to the amount of glucose given

Limited food intake when adequate food is available may be voluntary or the result of disease Lack of appetite is characteristic of many physical and mental ills and restriction of diets is a very common form of medical treatment The physician who prescribes diets must keep in mind the vitamins and other food factors and must make up with vitamin prepara tions the deficiencies in the foodstuffs allowed Many people not always the elderly, the poor or the notably peculiar, subject themselves to strange limitations of diet which may lead to deficiency disease A properly taken medical history includes an account of the patient's actual diet

Absorption or utilization of vitamins may be impaired in gastrointestinal hepatic or endocrine disease The use of mineral oil cathartics or adsorbing agents in therapy or the surgical removal of portions of the gastrointestinal tract may limit the ability to absorb vitamins as well as other foodstuffs

Excretion of vitamins may be accelerated as a result of excessive fluid output through skin or urinary tract *Destruction* of vitamins may occur excessively in the alimentary tract or in the body in abnormalities of the digestive secretions (e g achlorhydria) or as the result of poisoning with heavy metals or certain synthetic organic compounds (15)

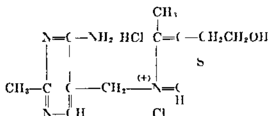
THE VITAMIN B COMPLEX

On account of the fundamental physiological importance of certain mem bers of this group we here abandon the traditional alphabetical order and give the group of B vitamins first consideration The term vitamin B in itself no longer has significance It was once applied to the crude material obtained from rice polishings which prevented the development of beriberi in man and of similar diseases in experimental fowl and other animals This substance, now known in its pure form as thiamine or vitamin B_1 is but one of a rather large group of substances which make up the vitamin B complex They are water soluble organic c ... nds which are necessary components of all living cells Their funct ... alone or as structural components of more c... ex molecules, is ... systems where they function as co-en ... substances ... up the B complex exist and have si... s in single ... s and in all the cells of all species ... nimals I ... never they

are produced adequately or even abundantly within the organism For such species they are, of course, not vitamins The concept of the B complex is anthropocentric, and is limited to those substances falling within this general category which are not synthesized by man or by common laboratory animals and therefore are dietary requirements for these species If one of these substances is synthesizable by a given organism, it can be termed, along with the true vitamins for that organism as an *essential metabolite*

Thiamine

Thiamine, known also as vitamin B_1 and in the European literature as aneurine, is the substance which was present in the early crude mixture obtained from rice bran which was effective in the prevention of polyneuritis in animals and the corresponding human disease, beriberi Thiamine

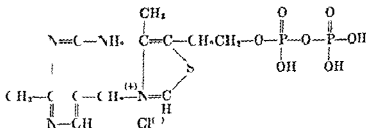


I Thiamine chloride hydrochloride

itself is 2,5 dimethyl 6 aminopyrimidine united with 4 methyl 5 hydroxyethylthiazol The formula shown in formula I is that of the chloride-hydrochloride, the form in which it is commonly marketed Thiamine functions as a part of the catalytic system of living cells in the form of its pyrophosphate ester, cocarboxylase This co enzyme is notably involved in the decarboxylation of pyruvic acid The chief biochemical lesion in beriberi is, therefore, the failure of pyruvic acid to enter the tricarboxylic acid cycle, in which it is normally oxidized Thiamine is also necessary in the tricarboxylic acid cycle itself at the step where alpha ketoglutaric acid is converted to succinic acid In thiamine deficiency, chemical analysis shows increased amounts of both pyruvic and lactic acid in the blood and in the urine as well as diminished amounts of thiamine Analysis for thiamine is more specific and more informative diagnostically The average concentration of thiamine in human blood is 3 4 micrograms per 100 ml, with a standard deviation of 1 1 micrograms (16) Thiamine is converted into cocarboxylase (formula II) within cells by the action of a phosphorylase, utilizing ATP as the source of phosphate Thiamine can enter and leave cells freely and appears in the urine provided the intake is adequate

Cocarboxylase once formed remains and functions within the cell and is not a normal urinary component In well nourished subjects the output of thiamine in the urine averages 230 micrograms per day Values below 150 micrograms are considered suboptimal, and less than 100 are indicative of a deficient intake (4) The minimal daily adult intake for prevention of beriberi is about 0 4 milligrams The daily intake advised by the National Research Council is 1 2 to 1 8 milligrams

Thiamine deficiency has been a common cause of heart disease in the United States (31) The cardiac disturbances can reasonably be attributed to the inability of the heart muscle to make adequate oxidative utilization of the normal metabolites pyruvic and lactic acids In early or mild deficiencies the heart rate is increased Later enlargement of the heart develops with decreased diastolic blood pressure and eventual congestive failure Electrocardiographic changes in beriberi are not specific Slowing



II Thiamine pyrophosphate or cocarboxylase

of the heart rate may occur in advanced cases but this is more commonly observed in experimental animals

The neuritis of thiamine deficiency starts with degeneration of the myelin sheaths which may be followed by fragmentation of axons The motor function of nerves may be depressed in varying degrees from barely perceptible muscular weakness to complete paralysis Disturbances of function of the sensory nerves are variable and often bizarre in their distribution There may be anesthesia hyperesthesia paresthesia or even severe cramp like pain Both motor and sensory neural disturbances usually appear first in the lower extremities and in mild or moderate cases are often limited to that area Advanced cases often show generalized edema—the so-called wet beriberi Alcoholism has been a frequent causative factor of thiamine deficiency in the United States Those patients who find themselves impelled into protracted periods of heavy drinking usually take but little food during these periods and that food is usually of poor nutritional value From this situation develops the typical "alcoholic polyneuritis" which is really an acute form of beriberi and if not too advanced responds well to thiamine therapy Even in advanced cases some useful degree of remyelini

zation and regeneration often occurs with adequate treatment. The American alcoholic population has demonstrated a notable decrease in beriberi and in other deficiency diseases referable to the B complex since the vitamin enrichment of white bread has become customary (9). A much greater incidence of beriberi unrelated to alcoholism is recorded in those oriental populations who are forced to rely mainly upon polished rice in their diet. Most of the oriental cases are chronic and relatively mild. The severity is increased in localities and at times of economic hardship. Raw fish and raw clams contain a thiaminase which may destroy the thiamine of foodstuffs during the early stages of the digestive process. This mechanism is of little significance in human nutrition but is economically important as the cause of the "Chastek" paralysis of ranch foxes fed on raw fish.

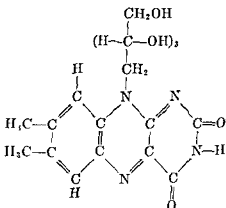
Of foods commonly used in human diet, yeast is the most abundant source of thiamine as well as of most of the other vitamins of the B group. Large amounts of yeast were used in the making of old fashioned homemade bread, providing adequate amounts of the B vitamins. Although modern baking methods do not use such large proportions of yeast, the deficiency is made up by the purposeful enrichment of bread with added thiamine along with other vitamins and valuable nutritive substances. Grains in their native state are excellent sources of thiamine with the highest concentration occurring in the germ. Of meats commonly used, pork is the best source, with about five times the concentration of thiamine as compared to beef or mutton. These latter meats, along with fish and fresh fruits, vegetables, and nuts, are significant dietary contributors of thiamine. Measurement of the thiamine content of foodstuffs can be accomplished biologically by the estimation of the amount required to prevent polyneuritis in experimental animals maintained on a deficient diet or by measurement of the growth of micro-organisms which require thiamine as a growth factor. A satisfactory chemical method of thiamine determination (5) involves the oxidation of thiamine to thiochrome in alkaline potassium ferricyanide solution. The concentration of thiochrome is measured by its fluorescence in ultraviolet light. A satisfactory colorimetric method depends upon the reaction of thiamine in alkaline medium with diazotized p-amino acetophenone (21). Thiamine in the dry form and in acid solution is stable to heat. It is less stable as the alkalinity of the solution is increased. These properties are responsible for varying degrees of destruction of thiamine in foodstuffs during cooking.

The toxicity of thiamine is low enough to render the administration of ordinary prophylactic or therapeutic doses a perfectly safe procedure. Intravenous dosages of thiamine hydrochloride in concentrations of 100 mgm per ml and in total dosage of 126 mgm per kgm of body weight have caused death in rabbits by paralysis of the respiratory center (11), and

at least one death apparently the result of intravenous overdosage with the same substance has occurred in man

Riboflavin

Riboflavin (formula III) is 6,7 dimethyl 9(*d* ribityl) isoalloxazine It is no longer commonly called by its original designations, vitamin B_2 or G *In the form of FAD (flavin adenine dinucleotide) it is the prosthetic group of the* flavin enzymes which are found in all animal cells Examples of such flavin enzymes which were considered in greater detail in Chapter 5 are cytochrome reductase, xanthine oxidase, and D amino acid oxidase *Other types of flavin enzymes occur in which the prosthetic group is ribo flavin phosphate*, for example, the L-amino acid oxidase of rat kidney



III Riboflavin

Rats placed on a diet deficient in riboflavin but otherwise adequate fail to grow They shed much or all of their fur, and develop *areas of symmetri cal dermatitis* A very characteristic sign is the ingrowth of blood vessels into the normally avascular cornea of the eye In human ariboflavinosis, as in the rat, the cornea becomes vascularized and may eventually become opaque *The tongue takes on a magenta color* and is often fissured Painful lesions develop at the corners of the mouth accompanied by hyperactivity ... deficiency is a

It is less frequently met with in the United States The underlying ... chemical lesion in riboflavin deficiency is, naturally, a failure of those oxida tions catalyzed by flavoprotein enzymes Thus, failure of xanthine oxidase activity can be demonstrated in the livers of riboflavin-deficient rats Also an over all deficiency of *riboflavin in the whole body* can be demonstrated

in deficient animals as compared to normally nourished controls. In human cases of riboflavin deficiency it is possible to demonstrate diminished urinary output of the substance. In the urines of normal individuals Cleland (4) found daily excretions of 130 to 800 micrograms of riboflavin with a mean value of 340. This compares well with the results of a similar series in which riboflavin was determined by a microbiological method (10), where the range was 67 to 980 micrograms with an average of 34[illegible]. The chemical method referred to, like most methods for riboflavin, depends upon the fluorescence of this substance under ultraviolet light. Microbiological assay is carried out by measurement of the growth rate of organisms such as *Lactobacillus casei* and *Leukonostoc mesenteroides* which require an external source of riboflavin.

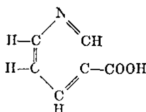
The National Research Council's recommendation for intake of riboflavin is from 1.5 to 1.8 milligrams per day. The experimentally determined minimal requirement for an adult male on a 2,200 Kcal. daily food intake is between 1.1 and 1.6 mgm. daily (13). As with most of the B vitamins, yeast is the richest source, supplying from 2 to 8 milligrams per 100 grams of dry material. Among other foods, milk is the best everyday source. Significant contributions of riboflavin are made to the diet by green leafy vegetables, eggs, meats and by most fruits and vegetables. Wheat germ is a rich source, which fact makes whole wheat products very satisfactory contributors of this vitamin to the diet. Riboflavin is quite stable to heat, but is destroyed by exposure to light. In addition to the dietary sources of riboflavin, some contribution is made by the production of this vitamin as a result of the action of intestinal bacteria. As far as human nutrition is concerned, the contribution is probably small. In cattle, sheep and goats, where bacterial action in the rumen takes place early in the digestive process and before any significant amount of absorption has occurred, bacterial production of this vitamin takes on a much greater significance. In cattle the output of riboflavin in the day's milk has been found to be as much as ten times the riboflavin of the day's diet (32). It should be pointed out in connection with all the B vitamins that while we consider them as deriving chiefly from plant sources, the plants may in turn derive them from bacteria and fungi resident in the soil and possibly symbiotically associated with the rootlets of the plants.

Measurement of very low levels of riboflavin can not be accomplished by the relatively crude methods used for urine or foodstuffs. By the use of a very sensitive fluorometer, the level in human blood serum of free riboflavin plus riboflavin phosphate has been found to be 0.8 micrograms per 100 ml., with a standard deviation of 0.05 micrograms. In addition to this, FAD was present in an amount corresponding to three times that figure. The riboflavin content of white cells and platelets in the same subjects

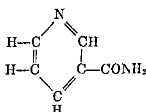
was 252 micrograms per 100 ml with a standard deviation of 11 micrograms. The corresponding figures for red cells were 22.4 micrograms per 100 ml with a standard deviation of 1.3 micrograms (1).

Nicotinic Acid

Following the rule of selecting the simplest effective structure as the vitamin *nicotinic acid* (formula IV) is named rather than its amide *nicotinamide* (formula V). Both of these are equally effective as vitamins. *Niacin* and *niacinamide* are synonyms for these two compounds. Nicotinamide is a structural component of two important coenzymes DPN and TPN. These two coenzymes have been found in every cell or tissue which has ever been brought under examination. They can be synthesized from nicotinic acid or nicotinamide by all animal cells and by almost all other cells (certain bacteria of the *Hemophilus* species can not put together the



IV Nicotinic acid



V Nicotinamide

pyridine ribose linkage). Most of the nicotinic acid in tissues is present as DPN and TPN.

The National Research Council's recommendation for daily intake of nicotinic acid is 12 to 18 mgm per day. Comparison of this figure with the results of numerous attempts to establish the actual human requirement indicates that the recommendation is probably above the minimum. Intakes of 30 mgm or more of nicotinic acid produce a definite pharmacological effect somewhat comparable to that of histamine. There is flushing particularly of the face and hands, sometimes with itching and burning. Aside from this apparently harmless effect, which is not observed with nicotinamide, these vitamins show no toxic effects until the dose is increased in experimental animals to amounts greater than one gram per kgm of body weight.

The human manifestation of nicotinic acid deficiency is *pellagra*, which is the most prevalent of the serious avitaminoses in the United States. As usually seen, pellagra is not the result of pure nicotinic acid deficiency but carries with it evidences of deficiency of the other B vitamins. The predisposing cause of pellagra is almost invariably alcoholism or poverty, or both. There is a seasonal variation in the incidence of pellagra in tem

perate climates which is out of phase with the seasonal changes in the supply of B vitamins. This has been explained (26) on the basis of seasonal differences in metabolic activity. Before the demonstration by Goldberger that pellagra was a disease of nutritional deficiency curable by feeding of yeast, the incidence of pellagra was high in prisons and asylums and particularly in regions where corn (*Zea mays*) meal was the dietary staple unmitigated by fresh meats or vegetables. The symptoms of pellagra have long been mnemonically described as "dermatitis, diarrhea, and dementia." The dermatitis tends to be symmetrical and to present sharply marked-off areas of red, thickened skin which later turn brown and scaly. It is likely to be restricted to areas which are exposed to light or to friction. The skin areas surrounding the external genitalia are particularly prone to pellagrous dermatitis, and may be affected without involvement of other areas. The tongue is red and swollen, later cracked and peeling. The diarrhea is both an early and a persistent manifestation and is one of the chief contributing causes of death in pellagrins. Achlorhydria and a macrocytic hyperchromic anemia often develop. Persistent nausea is a common symptom. The dementia of pellagra may range from the mildest of psychoneuroses to the most severe manic or stuporous states. The association of these psychotic manifestations with pellagra is verified by their prompt favorable response to therapy with nicotinic acid, usually within a week. Dosages of nicotinic acid in the therapy of pellagra may run as high as one gram per day, and are more effective if accompanied by the other components of the B complex. It is very difficult to develop nicotinic acid deficiency in rats. Monkeys develop experimental pellagra; pigs and dogs have frequently developed pellagra, or the canine equivalent "blacktongue," spontaneously as well as experimentally.

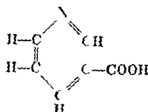
As it is for all the other B vitamins, yeast is a rich source of nicotinic acid. Among other foods, liver leads the list in nicotinic acid content. Good sources are meats, fish, eggs, whole wheat, unpolished rice, and peanuts. Nicotinic acid is not destroyed by ordinary cooking or by canning. There is considerable evidence that many animals, including man, can meet a part of the nicotinic acid requirement by synthesizing it with tryptophane as the starting substance (32). Pellagra-producing diets in man have been characterized by low content of tryptophane as well as of nicotinic acid. Maize is not only low in tryptophane but also contains substances definitely antagonistic to nicotinic acid.

Both nicotinic acid and nicotinamide are excreted in human urine along with N¹-methylnicotinamide (formula VII), which makes up about 20 per cent of the total excretion of nicotinic acid and its derivatives. The total excretion varies with the intake, and has been reported as high as 43 milligrams and as low as 1.7 mgm. per day in presumably well-nourished

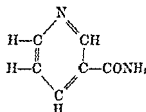
was 252 micrograms per 100 ml, with a standard deviation of 11 micrograms. The corresponding figures for red cells were 22 4 micrograms per 100 ml, with a standard deviation of 1 3 micrograms (1)

Nicotinic Acid

Following the rule of selecting the simplest effective structure as the vitamin *nicotinic acid* (formula IV) is named rather than its amide, *nicotinamide* (formula V) Both of these are equally effective as vitamins *Niacin* and *niacinamide* are synonyms for these two compounds Nicotinamide is a structural component of two important coenzymes, DPN and TPN These two coenzymes have been found in every cell or tissue which has ever been brought under examination They can be synthesized from nicotinic acid or nicotinamide by all animal cells and by almost all other cells (certain bacteria of the *Hemophilus* species can not put together the



IV Nicotinic acid



V Nicotinamide

pyridine ribose linkage) Most of the nicotinic acid in tissues is present as DPN and TPN

The National Research Council's recommendation for daily intake of nicotinic acid is 12 to 18 mgm per day Comparison of this figure with the results of numerous attempts to establish the actual human requirement indicates that the recommendation is probably above the minimum Intakes of 30 mgm or more of nicotinic acid produce a definite pharmacological effect somewhat comparable to that of histamine There is flushing particularly of the face and hands sometimes with itching and burning Aside from this apparently harmless effect, which is not observed with nicotinamide, these vitamins show no toxic effects until the dose is increased in experimental animals to amounts greater than one gram per kgm of body weight

The human manifestation of nicotinic acid deficiency is *pellagra* which is the most prevalent of the serious avitaminoses in the United States As usually seen pellagra is not the result of pure nicotinic acid deficiency, but carries with it evidences of deficiency of the other B vitamins The predisposing cause of pellagra is almost invariably alcoholism or poverty, or both There is a seasonal variation in the incidence of pellagra in tem

perate climates which is out of phase with the seasonal changes in the supply of B vitamins. This has been explained (26) on the basis of seasonal differences in metabolic activity. Before the demonstration by Goldberger that pellagra was a disease of nutritional deficiency curable by feeding of yeast, the incidence of pellagra was high in prisons and asylums and particularly in regions where corn (*Zea mays*) meal was the dietary staple, unmitigated by fresh meats or vegetables. The symptoms of pellagra have long been mnemonically described as "dermatitis, diarrhea, and dementia." The dermatitis tends to be symmetrical and to present sharply marked-off areas of red, thickened skin which later turn brown and scaly. It is likely to be restricted to areas which are exposed to light or to friction. The skin areas surrounding the external genitalia are particularly prone to pellagrous dermatitis, and may be affected without involvement of other areas. The tongue is red and swollen, later cracked and peeling. The diarrhea is both an early and a persistent manifestation and is one of the chief contributing causes of death in pellagrins. Achlorhydria and a macrocytic hyperchromic anemia often develop. Persistent nausea is a common symptom. The dementia of pellagra may range from the mildest of psychoneuroses to the most severe manic or stuporous states. The association of these psychotic manifestations with pellagra is verified by their prompt favorable response to therapy with nicotinic acid, usually within a week. Dosages of nicotinic acid in the therapy of pellagra may run as high as one gram per day, and are more effective if accompanied by the other components of the B complex. It is very difficult to develop nicotinic acid deficiency in rats. Monkeys develop experimental pellagra; pigs and dogs have frequently developed pellagra, or the canine equivalent "blacktongue," spontaneously as well as experimentally.

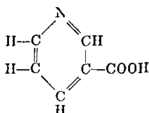
As it is for all the other B vitamins, yeast is a rich source of nicotinic acid. Among other foods, liver leads the list in nicotinic acid content. Good sources are meats, fish, eggs, whole wheat, unpolished rice, and peanuts. Nicotinic acid is not destroyed by ordinary cooking or by canning. There is considerable evidence that many animals, including man, can meet a part of the nicotinic acid requirement by synthesizing it with tryptophane as the starting substance (32). Pellagra-producing diets in man have been characterized by low content of tryptophane as well as of nicotinic acid. Maize is not only low in tryptophane but also contains substances definitely antagonistic to nicotinic acid.

Both nicotinic acid and nicotinamide are excreted in human urine along with N¹-methylnicotinamide (formula VII), which makes up about 20 per cent of the total excretion of nicotinic acid and its derivatives. The total excretion varies with the intake and has been reported as high as 15 milligrams and as low as 1.7 mgm. per day in presumably well nourished

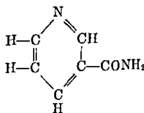
was 252 micrograms per 100 ml with a standard deviation of 11 micrograms. The corresponding figures for red cells were 22 4 micrograms per 100 ml with a standard deviation of 1 3 micrograms (1)

Nicotinic Acid

Following the rule of selecting the simplest effective structure as the vitamin *nicotinic acid* (formula IV) is named rather than its amide *nicotinamide* (formula V) Both of these are equally effective as vitamins *Niacin* and *niacinamide* are synonyms for these two compounds Nicotinamide is a structural component of two important coenzymes DPN and TPN These two coenzymes have been found in every cell or tissue which has ever been brought under examination They can be synthesized from nicotinic acid or nicotinamide by all animal cells and by almost all other cells (certain bacteria of the *Hemophilus* species can not put together the



IV Nicotinic acid



V Nicotinamide

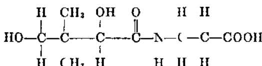
pyridine ribose linkage) Most of the nicotinic acid in tissues is present as DPN and TPN

The National Research Council s recommendation for daily intake of nicotinic acid is 12 to 18 mgm per day Comparison of this figure with the results of numerous attempts to establish the actual human requirement indicates that the recommendation is probably above the minimum Intakes of 30 mgm or more of nicotinic acid produce a definite pharmacological effect somewhat comparable to that of histamine There is flushing particularly of the face and hands sometimes with itching and burning Aside from this apparently harmless effect which is not observed with nicotinamide these vitamins show no toxic effects until the dose is increased in experimental animals to amounts greater than one gram per kgm of body weight

The human manifestation of nicotinic acid deficiency is *pellagra* which is the most prevalent of the serious avitaminoses in the United States As usually seen pellagra is not the result of pure nicotinic acid deficiency but carries with it evidences of deficiency of the other B vitamins The predisposing cause of pellagra is almost invariably alcoholism or poverty or both There is a seasonal variation in the incidence of pellagra in tem

for example the acetylation of sulfonamide drugs Williams (32) has suggested that the coenzyme may be involved in an acetylation at the 17 position of the steroid nucleus in the formation of adrenal cortical hormones This suggestion, if verified would link the function of pantothenic acid with the consistently observed adrenocortical failure in deficient rats Another physiologically significant process involving acetylation is the formation of acetylcholine The mediation of coenzyme A has been definitely established in this process From the viewpoint of chemical pathology, this finding is consistent with the therapeutic value of pantothenic acid in those deficiencies of other B vitamins characterized by neurological changes

Coenzyme A can be estimated quantitatively by a rather complex enzymatic procedure (32), and it can be shown that the pantothenic acid content of plant and animal tissues is accounted for almost completely by the coenzyme Quite a number of microorganisms are adaptable to the assay of pantothenic acid including yeast *Lactobacillus casei* and *Lactobacillus arabinosus* Assay methods involving the growth of chicks or rats



VII Pantothenic acid

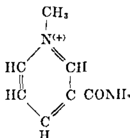
have been widely applied No suitable direct chemical method of analysis exists

Bacterial synthesis appears to play a very important part in the production of pantothenic acid The entire requirement in cattle seems to be met by bacterial production within the rumen, and up to 60 per cent of the requirement of rats appears to be supplied by microbial action in the cecum It is not known to what extent the human requirement is met from bacterial sources in the intestine, and actually there is considerable doubt as to the exact human requirement This dubious situation is partly explained by the fact already mentioned that no specific recognizable deficiency disease has been described in human subjects Williams (32) recommends a daily intake of 9 to 12 milligrams based on a total caloric intake of 2500 Kcal No toxic effects have been observed from large doses of pantothenic acid administered to man or to experimental animals The daily urinary output of pantothenic acid is in the neighborhood of 3 mgm Human blood contains about 30 micrograms per 100 ml

As with other B vitamins, yeast and liver are the most concentrated natural sources of pantothenic acid The supply in other foodstuffs is much the same as with the other B vitamins, except that wheat germ is

human subjects The mean value is 12 8 mgm per day (4) There is very little nicotinic acid demonstrable in blood plasma There is somewhat over one mgm per 100 ml of red blood cells, chiefly in the form of co enzymes The coenzymes, DPN and TPN, do not appear in the urine

The *microbiological assay utilizing a strain of Lactobacillus arabinosus* has been so far the most satisfactory of analytical procedures It is equally responsive, mol for mol, to nicotinic acid, nicotinamide, and the coenzymes A number of colorimetric methods have been proposed which lack the specificity of the microbiological assay N[1] methylnicotinamide in the urine can be measured relatively *simply by a fluorometric method* (5) Separation of nicotinic acid from nicotinamide is most easily accomplished by paper chromatography



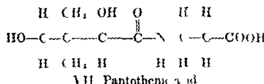
VI N[1] methylnicotinamide

Pantothenic Acid

The structure of this substance as shown in formula VII can be described as a dipeptide of beta alanine and a substituted butyric *acid No definite* human disease has been proved to result from pure deficiency of this vitamin The deficient diets, however, which lead to the development of beriberi, ariboflavinosis, and pellagra are also usually deficient in panto thenic acid A diminished concentration of pantothenic acid in the blood *has been frequently observed in these diseases* (32) Pantothenic acid has been found to be a useful adjunct in the treatment of these deficiencies and of the various manifestations of avitaminosis associated with alcoholism A very characteristic syndrome develops in rats maintained on a diet deficient in pantothenic acid There is parenchymatous damage to kidney and heart and particularly to the adrenals The external signs include dermatitis inflammation around the mouth and nose, and in dark colored rats graying of the hair The chief known function of pantothenic acid is the part that it plays in the make up of coenzyme A Coenzyme A is involved in the condensation of 2 carbon fragments whether derived from fat or from carbohydrate, with oxaloacetic acid to initiate the Krebs tri carboxylic acid cycle The same coenzyme is involved *in acetylations* as

for example the acetylation of sulfonamide drugs Williams (32) has suggested that the coenzyme may be involved in acetylation at the 17 position of the steroid nucleus in the formation of adrenal cortical hormones. This suggestion, if verified, would link the function of pantothenic acid with the consistently observed adrenocortical failure in deficient rats. Another physiologically significant process involving acetylation is the formation of acetylcholine. The mediation of coenzyme A has been definitely established in this process. From the viewpoint of chemical pathology, this finding is consistent with the therapeutic value of pantothenic acid in those deficiencies of other B vitamins characterized by neurological changes.

Coenzyme A can be estimated quantitatively by a rather complex enzymatic procedure (32) and it can be shown that the pantothenic acid content of plant and animal tissues is accounted for almost completely by the coenzyme. Quite a number of microorganisms are adaptable to the assay of pantothenic acid, including yeast, *Lactobacillus casei*, and *Lactobacillus arabinosus*. Assay methods involving the growth of chicks or rats



VII. Pantothenic acid

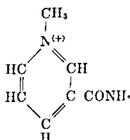
have been widely applied. No suitable direct chemical method of analysis exists.

Bacterial synthesis appears to play a very important part in the production of pantothenic acid. The entire requirement in cattle seems to be met by bacterial production within the rumen, and up to 60 per cent of the requirement of rats appears to be supplied by microbial action in the cecum. It is not known to what extent the human requirement is met from bacterial sources in the intestine, and actually there is considerable doubt as to the exact human requirement. This dubious situation is partly explained by the fact already mentioned that no specific recognizable deficiency disease has been described in human subjects. Williams (32) recommends a daily intake of 9 to 12 milligrams, based on a total caloric intake of 2,500 Kcal. No toxic effects have been observed from large doses of pantothenic acid administered to man or to experimental animals. The daily urinary output of pantothenic acid is in the neighborhood of 3 mgm. Human blood contains about 30 micrograms per 100 ml.

As with other B vitamins, yeast and liver are the most concentrated natural sources of pantothenic acid. The supply in other foodstuffs is much the same as with the other B vitamins, except that wheat germ is

human subjects The mean value is 12 8 mgm per day (4) There is very little nicotinic acid demonstrable in blood plasma There is somewhat over one mgm per 100 ml of red blood cells, chiefly in the form of co enzymes The coenzymes, DPN and TPN, do not appear in the urine

The microbiological assay utilizing a strain of *Lactobacillus arabinosus* has been so far the most satisfactory of analytical procedures It is equally responsive, mol for mol, to nicotinic acid, nicotinamide, and the coenzymes A number of colorimetric methods have been proposed which lack the specificity of the microbiological assay N¹ methylnicotinamide in the urine can be measured relatively simply by a fluorometric method (5) Separation of nicotinic acid from nicotinamide is most easily accomplished by paper chromatography



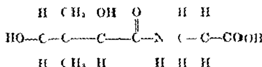
VI N¹ methylnicotinamide

Pantothenic Acid

The structure of this substance as shown in formula VII can be described as a dipeptide of beta alanine and a substituted butyric acid No definite human disease has been proved to result from pure deficiency of this vitamin The deficient diets, however, which lead to the development of beriberi, ariboflavinosis, and pellagra are also usually deficient in pantothenic acid A diminished concentration of pantothenic acid in the blood has been frequently observed in these diseases (32) Pantothenic acid has been found to be a useful adjunct in the treatment of these deficiencies and of the various manifestations of avitaminosis associated with alcoholism A very characteristic syndrome develops in rats maintained on a diet deficient in pantothenic acid There is parenchymatous damage to kidney and heart and particularly to the adrenals The external signs include dermatitis, inflammation around the mouth and nose and in dark colored rats, graying of the hair The chief known function of pantothenic acid is the part that it plays in the make up of coenzyme A Coenzyme A is involved in the condensation of 2 carbon fragments whether derived from fat or from carbohydrate, with oxaloacetic acid to initiate the Krebs tricarboxylic acid cycle The same coenzyme is involved in acetylations as

for example the acetylation of sulfonamide drugs. Williams (32) has suggested that the coenzyme may be involved in an acetylation at the 17 position of the steroid nucleus in the formation of adrenal cortical hormones. This suggestion, if verified, would link the function of pantothenic acid with the consistently observed adrenocortical failure in deficient rats. Another physiologically significant process involving acetylation is the formation of acetylcholine. The mediation of coenzyme A has been definitely established in this process. From the viewpoint of chemical pathology, this finding is consistent with the therapeutic value of pantothenic acid in those deficiencies of other B vitamins characterized by neurological changes.

Coenzyme A can be estimated quantitatively by a rather complex enzymatic procedure (32), and it can be shown that the pantothenic acid content of plant and animal tissues is accounted for almost completely by the coenzyme. Quite a number of microorganisms are adaptable to the assay of pantothenic acid, including yeast, *Lactobacillus casei*, and *Lactobacillus arabinosus*. Assay methods involving the growth of chicks or rats



VII Pantothenic acid

have been widely applied. No suitable direct chemical method of analysis exists.

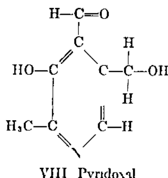
Bacterial synthesis appears to play a very important part in the production of pantothenic acid. The entire requirement in cattle seems to be met by bacterial production within the rumen, and up to 60 per cent of the requirement of rats appears to be supplied by microbial action in the cecum. It is not known to what extent the human requirement is met from bacterial sources in the intestine, and actually there is considerable doubt as to the exact human requirement. This dubious situation is partly explained by the fact already mentioned that no specific recognizable deficiency disease has been described in human subjects. Williams (32) recommends a daily intake of 9 to 12 milligrams, based on a total caloric intake of 2500 Kcal. No toxic effects have been observed from large doses of pantothenic acid administered to man or to experimental animals. The daily urinary output of pantothenic acid is in the neighborhood of 3 mgm. Human blood contains about 30 micrograms per 100 ml.

As with other B vitamins, yeast and liver are the most concentrated natural sources of pantothenic acid. The supply in other foodstuffs is much the same as with the other B vitamins, except that wheat germ is

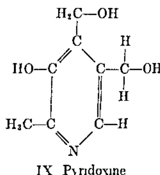
not a satisfactory source Pantothenic acid withstands moist heat in neutral solutions, but is rapidly hydrolyzed at higher pH values

Pyridoxal

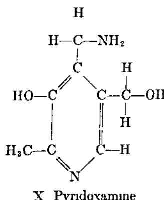
From the point of view of human nutrition *pyridoxal* (formula VIII), *pyridoxine* (formula IX), and *pyridoxamine* (formula X) are qualitatively and quantitatively equivalent These substances together constitute what



VIII Pyridoxal



IX Pyridoxine



X Pyridoxamine

was formerly known as vitamin B_6 Of this group pyridoxine is the form most abundant in cereal sources Pyridoxal phosphate and pyridoxamine phosphate are the metabolically active forms in which this vitamin occurs in cells and tissues In mammalian tissue pyridoxamine phosphate has no known significance while the chief function of pyridoxal phosphate is as a coenzyme for transaminases (see page 538) The same coenzyme is much more involved in bacterial metabolic reactions, particularly in the de carboxylation of certain amino acids Although such decarboxylations can be demonstrated in mammalian tissue, they do not take place there to any notable extent In bacteria, tyrosine, lysine, ornithine, and arginine are often decarboxylated to form the respective amines, tyramine, cadaverine,

putrescine, and argamine In a similar fashion, glutamic acid forms gamma-aminobutyric acid, and dihydroxyphenylalanine (dopa) forms 3,4-dihydroxyphenylethylamine The last-mentioned reaction becomes important in human physiology as well as in bacterial metabolism since the substance formed is a normal precursor of adrenalin The other reactions are significant to the human in that they take place by bacterial action in the intestine

The biochemical lesions which would be expected to occur in vitamin B_6 deficiency can be demonstrated in experimental animals Transamination can be shown to be deficient and abnormalities in the metabolism of tryptophane, particularly its conversion to nicotinic acid, can be demonstrated No characteristic single disease has been described in man which can be attributed solely to deficiency in this group of vitamins Lack of the B_6 group seems quite definitely involved in the symptoms observed in pellagra and beriberi, persistent neurological disturbances have responded well to therapy with pyridoxine, as have neurological symptoms associated with diseases not primarily of nutritional origin There is some controversy as to whether the lesions in the corners of the mouth, already described as characteristic of riboflavin deficiency, may not be the result of deficiency of pyridoxine and its relatives Pyridoxine deficiency in the young rat is characterized by *acrodynia*, which is a dermatitis obviously painful to the animal, developing chiefly on the paws, tail ears, nose, and mouth Rats and other experimental animals may develop epileptiform attacks as a result of restriction of the B_6 group Pyridoxine, however has not proven to be a reliable drug in the treatment of epilepsy in man

A number of colorimetric methods have been proposed for the determination of the B_6 group In general, these methods have proved non specific in distinguishing one member of the group from another, and all of these substances from other color reactive substances in foods and tissues Methods based upon the prevention of acrodynia in young rats on a vitamin B_6 deficient diet have been widely used Microbiological assay is carried out using yeast, *Neurospora*, or *Streptococcus faecalis* as the test organism

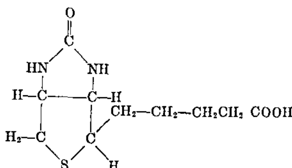
Since attempts to deplete human subjects of the B_6 group to the extent that a deficiency disease is produced have been unsuccessful, it is impossible to state with exactness the human requirement for these vitamins An intake of 1 5 milligrams per day has been recommended (32) No toxic effects of overdosage have been reported in man Toxic reactions have been produced in animals only with enormous overdosages of orders of magnitude of 3 grams per kgm of body weight, or more Although all three forms of vitamin B_6 may be demonstrated in the urine, these substances are excreted chiefly in the form of their metabolic product, 4 pyridoxic acid The daily excretion of pyridoxine in all forms is less than one milligram

Effective dietary sources of the vitamin B_6 group are in general similar to those of the other B vitamins Whole grain cereals, fresh meats fresh vegetables and milk are the major sources in the human diet

Biotin

Biotin is a rather curious member of the B complex because it is produced so abundantly by the synthetic action of intestinal flora that pure nutritional deficiency of biotin is not only seldom observed but difficult to produce Like the other members of the B group, it has been found to be a constituent of all cells where its presence has been investigated It is also of interest that two vitamins, originally thought different were later shown to be identical with a single substance, biotin

In 1901, Wildiers observed that a substance found in beer wort and in



XI Biotin

growing yeast cultures was required to bring about the growth of yeast in an otherwise purely synthetic medium Note that this substance is produced by growing yeast, although it is also a necessity for yeast growth Wildiers called this substance *bios* Considerable effort was expended in the isolation and analysis of bios, it was eventually found to consist of several components, one of which was isolated in 1935 and named biotin

The other experimental pathway leading to the discovery of biotin began with the observation by Bateman in 1916 of 'egg white injury" Raw egg white was found to contain a protein designated as *avidin* which combined irreversibly with a previously unrecognized vitamin which was for a time, designated as vitamin H Deficiency of this vitamin could be produced effectively only by egg white injury, which was characterized in rats by dermatitis, loss of hair, baldness around the eyes producing the so called 'spectacle eyes", and eventually emaciation and death About ten years later Boas found that a diet high in certain foods such as liver kidneys, egg yolk, yeast, or milk would protect animals against egg white injury The identification of so called vitamin H with biotin was accom

plished in 1940, and in 1942 the formula for biotin was tentatively proposed Later a slightly modified formula was confirmed by synthesis (formula XI) Also in that same year, 1942, biotin deficiency was reported in 4 human subjects who had been taking about 30 per cent of their total calories in the form of egg white over a period of 7 to 8 weeks (29) These men and women showed a striking ashy pallor, a dry skin with scaling, extreme lassitude and somnolence muscular pains and failure of appetite Relief from these symptoms took place within a few days after injections of biotin, 150 to 300 micrograms per day

Assay of biotin (32) is accomplished most satisfactorily by methods involving either the relief of egg white injury in experimental animals or the growth of yeast or of lactic acid bacteria Chemical methods are unsatisfactory because of the remarkably low concentrations in which biotin occurs in most foodstuffs, cells, and body fluids The biotin required in mammalian nutrition has its most important origin in bacterial action The production of biotin deficiency requires either the administration of raw egg white or the inhibition of bacterial action in the intestine of the experimental animal or subject by the administration of an antibacterial substance such as one of the sulfonamide drugs Men, cattle, and rats all excrete more biotin than is contained in their diet It appears probable that all micro-organisms require biotin, and that most of them are capable of synthesizing it When such organisms are grown in a biotin free medium, their rate of growth is limited by the rate of biotin synthesis

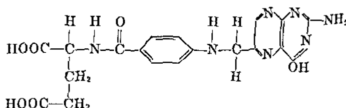
Numerous functions have been assigned to biotin containing coenzymes in the metabolism of yeast and other micro-organisms In man and other animals, biotin appears to be associated with the early stages of the tricarboxylic acid cycle and the fixation of carbon dioxide The two definite reactions in which it appears chiefly to be involved are the reversible decarboxylations of oxaloacetic acid and oxalosuccinic acid yielding CO_2 and respectively, pyruvic acid and alpha ketoglutaric acid The actual coenzyme has not been isolated, therefore the evidence for this relationship depends upon the ability of oxaloacetic acid or aspartic acid to replace the required biotin in micro organisms plus the failure of tissues lacking in biotin to carry out the metabolism of pyruvic acid

On account of the difficulty of completely eliminating intestinal synthesis of biotin in human experimentation, it has not been possible exactly to evaluate the human requirement Estimates have varied from as little as 2 micrograms to as much as 300 micrograms a day The average dietary intake appears to be in the neighborhood of somewhat less than 50 micrograms per day, the urinary output, slightly less than this, but the fecal output, on account of intestinal synthesis, is from two to six times the intake As might be expected, biotin is widely distributed in nearly all

types of naturally occurring foodstuffs Concentrations in foodstuffs are usually low, the richest sources being yeast, egg yolk, liver, and kidney

Folic Acid

This substance, known also as pteroylglutamic acid, is shown in formula XII This substance is the simplest compound which will carry out several separate vitamin functions which had previously been assigned to separate substances Folic acid by itself, or in the form of complexes, appears to be required for the growth of *Lactobacillus casei* and certain other bacteria Prior to the identification of folic acid, such growth promoting substances had been isolated from yeast, and from liver, and later from spinach and other leaves Since certain purines and pyrimidines can substitute for folic acid, and since thymine is the most effective of these, the hypothesis was proposed that folic acid forms, or is, a necessary coenzyme for the synthesis of thymine or a closely related compound Later another *L casei*



XII Folic acid (pteroylglutamic acid)

factor was isolated the so called fermentation *L casei* factor from a bacillus which sometimes contaminated molds used in the production of riboflavin This compound has turned out to be pteroyl triglutamic acid Folic acid has also been identified with "vitamin M" a substance present in certain yeast products which would cure the anemia developed in monkeys fed a diet containing adequate protein, minerals, fatty acids, along with vitamins A, B_1 C, and D Another presumed vitamin, known as "vitamin B_c", was found to be identical with the hydrolyzed *L casei* factor, its physiological effect was to prevent the macrocytic, hyperchromic anemia and failure of growth which resulted in chicks from lack of this particular B vitamin Folic acid and the *L casei* factor have been effective in the treatment of the macrocytic anemia of pregnancy, of nutritional anemias of tropical and non tropical sprue and also in the treatment of pernicious anemia, but in this last disease, folic acid and its relatives are not as effective as liver extract or the later discovered vitamin B_{12} The various folic acid conjugates and pteroyl polyglutamic acids, including the *L casei* factors from yeast and other microorganisms have been reviewed in detail by Sargent (25) The physiological activity of the conjugates is usually

increased by hydrolysis to folic acid, which is accomplished by an enzyme (vitamin B_c conjugase) present in many tissues These conjugates are widely distributed in nature, their best sources are spinach and liver among articles of customary human diet Lampen and Jones ... demonstrated that the ... certain organ... sms depends ... para-aminobenzoic acid as a substrate for the synthesis of folic acid In these particular bacterial species, folic acid antagonizes the antibacterial action of the sulfonamides noncompetitively Para aminobenzoic acid, on the other hand inhibits the action of the sulfonamides upon these species competitively However, this mechanism should not be interpreted as the only one involved in the antibacterial effect of the sulfonamide drugs Non competitive antagonism by folic acid could not be demonstrated in *E coli*, *S aureus* or *Diplococcus pneumoniae* If a micro organism does ... synthes... it ob... ... to inhibit this synth... ... in general, require folic acid and resemble the folic acid requiring bacteria in that they do not possess this particular metabolic process with which the sulfonamides interfere Folic acid deficiency can be induced in animals or in man by administration of ***folic acid antagonists*** such as 4 amino folic acid (aminopterin) or 4 amino N^{10} methyl folic acid (amethopterin) Such deficiency is characterized by leukopenia aplastic anemia, and ulceration of mucous membranes It is postulated that the folic acid antagonists compete with folic acid to enter a mechanism which normally forms a metabolic product such as the *citrovorum factor*, which exceeds folic acid in biological effect by a factor ... to the test ... of some ... the ant... ... acid, but respond to citrovorum factor The normal conversion of folic acid to citrovorum factor requires ascorbic acid (7) The citrovorum factor, and also a similar and possibly identical substance, *folinic acid*, are effective in the same types of anemia which respond to folic acid The citrovorum factor is formed metabolically from folic acid in rats and men and is excreted in the urine Synthesis of the citrovorum factor from folic acid has been accomplished *in vitro*, but the structure has not at the time of this writing been reported

Folic acid is another vitamin which we obtain in large measure by its production in the intestine by the action of bacteria Daily human oral intakes range from about 40 micrograms to somewhat more than double that figure, somewhat less than 5 micrograms is put out daily in the urine, but 200 to 300 micrograms are present in the feces In bacterial metabolism,

folic acid is quite definitely involved in reactions involving the introduction of a single carbon atom In cultures of certain bacteria including *E coli* when bacterial growth has been inhibited by the presence of sulfonamides a complex imidazole compound accumulates This compound has a structure such that if it could condense directly with a 1 carbon fragment it would form hypoxanthine It has been presumed that in carrying out this particular function folic acid forms a formyl derivative This substance is even more active than folic acid itself in opposing the action of certain inhibitors of bacterial growth

The human dietary requirement of folic acid and related compounds has not been definitely established on account of the contribution of intestinal bacterial synthesis Folic acid appears to be quite non toxic in any reasonable dosage for any of the laboratory animals Fresh green leafy vegetables and liver are the best food sources

Vitamin B_{12}

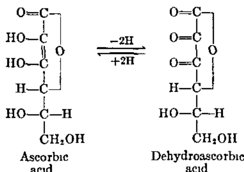
Crystalline vitamin B_{12} or *cyanocobalamine* can be obtained from liver and in larger yield from *Streptomyces griseus* as dark red needles with a minimum molecular weight of about 1300 based upon their 4 5 per cent content of cobalt The solid form is stable as are solutions at neutral or slightly acid pH The full structure has not yet been worked out but among the hydrolysis products are 5 6 dimethylbenzimidazole phosphoric acid ammonia and two unidentified fractions one of which is still of high molecular weight and contains cobalt There are at least three substances closely resembling each other making up a group of vitamins B_{12} all with similar biological activity There are also conjugated forms of vitamin B_{12} with peptides or proteins

The chief medical interest of vitamin B_{12} lies in the fact that it is more effective than any other substance in the treatment of pernicious anemia For adequate effect the vitamin must be given parenterally for reasons which will be immediately explained Pernicious anemia can be considered as the deficiency disease resulting from failure in the human to absorb vitamin B_{12} The failure is the result of the lack of a thermolabile *intrinsic factor* in the gastric juice This intrinsic factor was demonstrated in normal human gastric juice by Castle (2) and has not been isolated nor chemically characterized beyond the suggestions that it is associated with the glandular mucoprotein of the stomach and may be a proteolytic enzyme, distinct from pepsin The crucial test for its presence involves the incubation of meat with gastric juice Meat incubated with normal gastric juice and fed to patients with pernicious anemia produces a remission Meat incubated with the gastric juice of pernicious anemia patients has no effect The intrinsic factor of normal gastric juice was postulated by Castle

With blackening of the peel and fermentation of the pulp, the ascorbic acid content diminishes (30) Ascorbic acid is commercially synthesized from glucose, and is available in pure solid form Ascorbic acid, in equilibrium with its first oxidation product, dehydroascorbic acid, is the only natural substance which will protect against scurvy A few synthetic homologues and stereoisomers possess some protective power

Scurvy or scorbutus has been known for centuries, and its association with famines and with long sea voyages recognized In the United States scurvy occurs most commonly in children 6 months to 2 years of age, who have been bottle fed without supplementation with vitamin C Scurvy is also a threat to those persons, usually elderly, who live alone and prepare only the simplest possible meals for themselves

Scurvy in experimental animals and in premature infants is charac-



Ascorbic acid

Dehydroascorbic acid

XIII

terized by incomplete oxidative metabolism of tyrosine, indicated by the presence in the urine of tyrosine itself and intermediates such as p hydroxyphenylpyruvic acid and homogentisic acid This defect is remedied by small doses of ascorbic acid, but is also remedied by administration of folic acid, which does not protect against the other manifestations of scurvy ACTH is also effective in normalizing the metabolism of tyrosine in premature infants (18) It will be recalled that ascorbic acid is necessary for conversion of folic acid to citrovorum factor A more damaging metabolic 'fect in scurvy is the failure of production of the intercellular materials connective and supporting tissue (33), including both collagen and opolysaccharides (23) In clinical or experimental scurvy, capillary ige is a characteristic sign, brought about by the lack of interbinding substances In growing bones or healing fractures, forma nic matrix is suppressed with resulting deformities and failure ion, although calcium metabolism is not primarily affected ls is delayed through failure of formation of scar tissue

although it is used therapeutically in the treatment of a deficiency disease, alcoholic fatty liver Inositol is a component of Wildiers' bios along with biotin, and has been shown to be required by numerous microbial species It is certainly an essential structural component in man and other animals, forming a part of the phosphoinositides of liver, nervous system, and other tissues It is synthesized in the intestine and possibly in the body, and no specific deficiency disease has been attributed to lack of inositol in the human diet *Inositol* may be considered a B vitamin only by greatly stretching the classification

Para aminobenzoic acid has also been listed among the B vitamins, but has no known function in animals other than as a component of folic acid, and can not substitute for folic acid in all the functions of the latter substance

VITAMIN C

Ascorbic acid, a sugar acid which is the only substance properly designated as vitamin C, has some points in common with the B vitamins Like them, it is water soluble and is present consistently in the cells of mature plants and animals, but the concentrations of ascorbic acid in seeds of plants and embryos of animals are often found to be zero by our most sensitive methods Furthermore, the distribution of ascorbic acid among micro organisms, including protozoa, is sparse and erratic, indicating no uniformly significant function

The formula of ascorbic acid is shown here (formula XIII) as it occurs in natural sources, in equilibrium with its oxidized form, *dehydroascorbic acid* The reduced form predominates

The rich dietary sources of ascorbic acid, available for human use, include the citrus fruits, berries, green vegetables apples, and with less richness most other fruits and vegetables Meats in general contain suboptimal amounts of vitamin C, although it is present in all animal tissues Ascorbic acid can be crystallized as odorless, colorless plates, M P 192°C It is very soluble in water, insoluble in the fat solvents The crystals are stable, solutions are not Oxidation occurs on exposure of neutral or alkaline solutions or foodstuffs to air

The oxidative inactivation of ascorbic acid is accelerated by heat and by the presence of catalytic traces of copper Acidic foodstuffs, including most fruits, hold their vitamin C content well during normal periods of storage Some non acidic vegetables, such as potatoes, cabbage, and turnips, are customarily stored actually in the living state and keep their ascorbic acid content at a significant level Bananas maintain their respiration during storage and ripening and preserve a concentration of about 10 mgm ascorbic acid per 100 grams up to the stage where half of the peel is brown

mgm This minimum should be trebled to provide a reasonable factor of safety The National Research Council recommendation of 75 mgm is excessive, but in no sense harmful No toxic effects of ascorbic acid have ever been recorded either for guinea pigs or men other than a mild diuresis following enormous dosages The ascorbic acid of human blood plasma is 1 0 to 1 4 mgm per 100 ml when the subject is "saturated" with the vitamin (20), which means that any excess over this level is excreted in the urine Some ascorbic acid is excreted, however, at lower plasma levels A plasma level of 0 6 mgm or more can be considered evidence of adequate vitamin C supply In deprivation experiments, the ascorbic acid of the white blood cells, normally over 20 mgm per 100 ml, approaches or equals zero before signs of scurvy appear "Saturation" requires a dosage of about 100 mgm daily In the feeding of young infants, it is customary to start at the age of two weeks with 30 ml of orange juice daily and to raise the intake to 60 ml daily at the age of three months Thirty ml of fresh orange juice contains approximately 18 mgm of ascorbic acid

The body under stress requires more ascorbic acid It is characteristic of infectious disease that the plasma ascorbic acid is at a low level and that greater intakes are required to produce saturation (8) Similar findings have been reported in patients with burns and with fractures This observation has been extended in experimental animals by the demonstration of depletion of the ascorbic acid of the adrenal under stress provided the adenohypophysis is present and intact The conclusion that ascorbic acid is required and used in the synthesis of cortical steroids is not however consistent with the continued production of steroids by scorbutic guinea pigs whose adrenals are depleted of ascorbic acid (27)

Analysis for ascorbic acid is frequently done by titration with 2 6 dichlorophenol indophenol, which acts both as oxidizing agent and redox indicator, being blue in the oxidized form and colorless when reduced This titration must be carried out in solutions more acid than pH 5 5 since ascorbic acid is autoxidizable in more basic solutions This titration is of course not specific for ascorbic acid but measures the totality of substances present and capable of reducing the blue dye Such reducing substances are not significantly present in urine or blood plasma A more specific colorimetric analytical method involves the formation of a colored hydrazone of dehydroascorbic acid with 2 4-dinitrophenylhydrazine (5)

FLAVONES AND COUMARINS

The designation vitamin P is sometimes given to a rather large group of substances of plant origin which act to increase the strength of capillaries The discovery of this group of compounds grew from the observation that purified preparations of vitamin C were ineffective in the control of bleed

Bleeding of the gums is an early and classical symptom Bleeding under the skin or conjunctiva leads to visible signs Internal hemorrhages may become quite extensive before causing such symptoms as bloody stools or painful joints

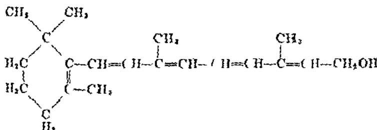
An experimenter (6) subsisted for six months on a diet containing no vitamin C The ascorbic acid of his plasma reached zero after 41 days and remained there The first sign observed referable to scurvy was the development of perifollicular hyperkeratotic papules on the skin of the buttocks and calves The papules contained ingrown hairs, which were frequently fragmented This sign had previously been considered a specific manifestation of vitamin A deficiency, but the experimenter had been taking a minimum of 30 000 I U of vitamin A daily, and his blood level of vitamin A was found normal The papules appeared on the 135th day of the diet at which time the ascorbic acid content of the white blood cells was zero After 161 days, perifollicular hemorrhages were observed in the skin During the entire experiment no bleeding of the gums occurred which can perhaps be explained by good oral hygiene There was no anemia and no increase of capillary fragility as measured by standard clinical tests Small hemorrhages did appear in the skin of the lower extremities after a long period of standing A surgical wound was made in the right mid back of the experimenter after 3 months on the deficient diet Biopsy ten days later demonstrated normal healing After 182 days on the diet a similar surgical wound was made in the left mid back Biopsy ten days later demonstrated no healing except in the skin Microscopic examination of sections showed newly formed fibroblasts but no intercellular substance between them Ingrowth of capillaries was lacking After the biopsy, the diet was maintained but one gram of ascorbic acid was given intravenously each day Ten days later another biopsy demonstrated healing with intercellular substance being formed and capillaries growing in Subjectively the experimenter felt tired and weak after the third month of the diet, and increasingly so until the termination of the deficiency All signs and symptoms cleared rapidly after daily vitamin C injections were given

Others have repeated these observations on other volunteer human subjects and have for the most part confirmed the findings outlined above

made worse Not all subjects have escaped without swollen gums Delayed healing of wounds has been repeatedly confirmed Many subjects do not report fatigue or weakness

All plants and most animals can synthesize their own vitamin C It is a true vitamin only for primates and guinea pigs The guinea pig requires one or two mgm per day, and man requires a bare minimum of about 10

by Levitan (19) along with a consideration of the application of the vitamins P in the treatment of a number of human diseases characterized by capillary fragility. Not only are the theories of vitamin P action in dispute, but even the facts concerning its action are not matters of uniform agreement. States of pure vitamin P deficiency have been reported in laboratory animals with resulting capillary fragility even though their intake of ascorbic acid was adequate. Such capillary fragility responded favorably to the administration of vitamin P. Deficiency of vitamin P has been described in the human, such cases showing response to vitamin P and no favorable effect from ascorbic acid. This work has not been fully confirmed.



XVI Vitamin A

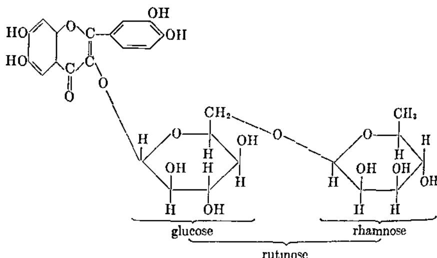
VITAMIN A

All vitamins so far discussed are classified as water soluble although riboflavin is actually rather sparingly soluble in aqueous solutions. With vitamin A we begin the study of the fat-soluble vitamins, which occur in the lipid portions of the foods in which they are found and which can be extracted with organic solvents. At least two substances possess what we are about to describe as vitamin A activity but the substance known as vitamin A_1 is primarily significant in all mammals and in salt water fish. This substance we will designate as vitamin A, neglecting vitamin A_2 which appears to have a physiological function only in the tissues of fresh water fish, and neglecting neovitamin A which is a *cis* isomer of vitamin A_1 with identical effects.

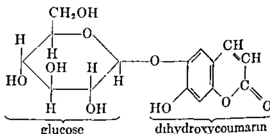
Vitamin A is an alcohol with the structure shown in formula XVI. As an alcohol, it can exist free or in the form of fatty acid esters. The cyclic portion is a beta ionone ring. If the terminal CH_2OH group is changed to CH and a double bond is left hanging free we now have half a molecule of the substance beta carotene. Ingested beta carotene is converted into vitamin A in the intestinal wall and possibly in the liver presumably by an oxidative cleavage at the central double bond forming first an aldehyde group and then the alcohol by reduction. Thus beta-carotene (formula

ing in vascular purpura, a disease in which capillary fragility is increased but that control was established by the use of crude and unpurified vitamin C preparations The first material which was definitely shown to exert vitamin P activity was a yellow preparation called *citrin*, obtained from fruits Citrin turned out to be a mixture of several active flavone derivatives

Flavone is usually considered to be the parent substance of the P vita



XIV Rutin



XV Esculin

mins Some of the active substances are substituted flavones, as for example rutin (formula XIV) Other substances with vitamin P activity have only a part of the flavone structure and are more properly designated as coumarins An example of this group is esculin (formula XV) which is about five hundred times as active as citrin All the P vitamins are of plant origin Rutin is obtained from buckwheat and esculin from chestnuts Not all flavone or coumarin derivatives show vitamin P activity

No unified or generally acceptable theory of the action of vitamin P has yet been proposed Several possible theories of this action are discussed

applied to blood plasma, utilizing the reaction of vitamin A with activated glycerol dichlorohydrin (28)

Sources. As has been suggested already, vitamin A itself appears in foods of animal origin The oils extracted from the livers of certain fish, particularly the cod, the halibut, and the shark, are the most concentrated sources of vitamin A Egg yolks, milk, and butter are sources which are more common in the American diet Oleomargarines sold for household use are customarily fortified with vitamin A Dietary sources of the carotenes are the leafy vegetables such as lettuce and cabbage, particularly the outer and greener leaves Green stalks such as asparagus and celery, and green seeds such as peas and green beans have high provitamin content, yellow vegetables and yellow fruits are uniformly valuable and rich sources In general, the grains are not very satisfactory in supplying these provitamins but yellow maize is an important exception

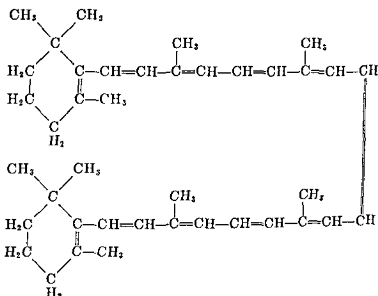
Pure beta-carotene has been accepted as the basis of the international unit of vitamin A The unit is defined as the biological activity equal to that of 0 6 micrograms of beta carotene The international standard preparation of vitamin A contains 0 3 milligrams of beta carotene dissolved in one gram of an inert vegetable oil Each 2 milligrams of the international standard preparation will therefore contain one international unit of vitamin A activity

Absorption and storage. Vitamin A is absorbed by the same mechanisms as the lipids of foodstuffs The presence of bile in the intestine is necessary for such absorption Vitamin A is stored in considerable amount in the liver, concentrations between 100 and 900 international units of vitamin A per gram having been found in 80 per cent of the livers of a series of 71 healthy individuals whose death was sudden and accidental (14) Absorption of the carotenes from the intestine is less efficient than that of the vitamin A alcohols Not all of the carotenes are converted to vitamin A Some of the color of skin and of blood plasma is from normal content of carotene Vitamin A deficiency may occur not only from lack of the vitamin or its precursors in the diet, but also from failure of absorption from the intestine as by excessive ingestion of mineral oil, or from failure of conversion of carotenes or storage of vitamin A or both by a diseased liver The mean concentration of vitamin A in the blood plasma of adequately nourished men and women is close to 120 international units per 100 ml values for total carotenoids are higher and more variable

Physiological functions. Vitamin A is concerned with growth, with the maintenance of epithelial tissues, and with vision The first evidence for its existence was presented in 1913 by two independent teams of workers certain natural fats and oils would stimulate growth in rats while other fats and oils with similar triglyceride composition would not The necessity

XVII) can properly be designated as a provitamin A, since two molecules of vitamin A can be obtained from one molecule of the parent carotene Alpha carotene, gamma carotene, and the cryptoxanthines found in egg yolk and yellow corn are also provitamins A, but can yield only one molecule of vitamin A per molecule of original substance In general vitamin A occurs in animal foods and the carotenes in plant foods Butterfat, however, contains both

Vitamin A has no significant solubility in water, is soluble in fats and fat solvents, and is stable to heat in the absence of air or oxygen The vita



XVII β Carotene

min A of cod liver oil is destroyed by heating the oil to the boiling point of water for 12 hours while air is being passed through the oil This treatment does not destroy vitamin D The provitamins or carotenoids have similar properties as far as solubility and stability are concerned The ordinary processes of cooking and canning do not cause serious loss The preservation of foods by freezing does not impair their vitamin A content, but dehydration of foods tends to cause appreciable loss of vitamin A

Assay of vitamin A may be biological, involving measurements of the growth of rats, or by physical or chemical methods Spectrophotometry is satisfactory for fairly concentrated preparations, and is based upon absorption maxima at 610 to 620 millimicrons for A_1 and 692 and 696 millimicrons for A_2 Colorimetric analysis is very sensitive, and can be

applied to blood plasma, utilizing the reacti n of vitamin A with activated glycerol dichlorohydrin (28)

Sources. As has been suggested already vitamin A itself appears in foods of animal origin The oils extracted from th livers of certain fish particularly the cod the halibut and the shark are the most concentrated sources of vitamin A Egg yolks milk and butter are sources which are more common in the American diet Oleomargarines sold for househ ld use are customarily fortified with vitamin A Dietary sources of the carotenes are the leafy vegetables such as lettuce and cabbage, particularly the outer and greener leaves Green talks such as a paragus and celery, and green seeds such as peas and green beans have high provitamin content, yellow vegetables and yellow fruits are uniformly valuable and rich sources In general, the grains are *not very satisfactory* in supplying these provitamins but yellow maize is an imp rtant excepti n

Pure beta carotene has been accepted the basis of the international unit of vitamin A The unit is defined as the biological activity equal to that of 0 6 micrograms of beta-carotene The international standard preparation of vitamin A contains 0 3 milligrams of beta carotene dissolved in one gram of an inert vegetable oil Each 2 milligrams of the international standard preparation will therefore contain one international unit of vitamin A activity

Absorption and storage Vitamin A is absorbed by the same mechanisms as the lipids of foodstuffs The presence of bile in the intestine is necessary for such absorption Vitamin A is stored in considerabl [illegible] in the liver, concentrations between 100 [illegible] vitamin A per gram [illegible] of 71 healthy [illegible] Absorption of [illegible] less efficient than that of the vitamin A a [illegible] of all of the carotenes are converted to vitamin A Some of the color of skin and of blood plasma is from normal content of carotene Vitamin A deficiency may occur not only from lack of the vitamin or its precursors in the diet but also from failure of absorption from the intestine, as by excessive ingestion of mineral oil or from failure of conversion of carotenes or storage of vitamin A or both by a diseased liver The mean concentration of vitamin A in the blood plasma of adequately nourished men and women is close to 120 international units per 100 ml, values for total carotenoids are higher and more variable

Physiological functions Vitamin A is concerned with growth [illegible] the maintenance of epithelial tissues, and with [illegible] for its existence was present [illegible] certain natural fats and [illegible] other fats and oils with similar [illegible] would not The necessity

of vitamin A for growth has been repeatedly confirmed The growth of the skeleton—not calcification, which is related to the vitamins D but the actual growth of the organic matrix—is first affected by the lack of vitamins A in a young animal Growth of soft tissues is less promptly checked, so that brain and cord may grow more rapidly than their bony cases and thus suffer damage by constriction In animals deficient in vitamins A at any age, epithelial cells tend to atrophy and to be replaced from the basal cells by a stratified horny epithelium, high in keratin content Lachrymal glands and conjunctiva are particularly sensitive to deficiency of the vitamins A, so that in experimentally deficient animals and in severe cases of human deficiency a dry-eyed state, *xerophthalmia*, is observed The thick and dry conjunctiva is susceptible to infection and to mechanical damage In a more advanced stage of deficiency, the cornea wrinkles and shrinks (*keratomalacia*) and may ulcerate and perforate Other epithelial tissues which are affected in severe deficiency include the skin, salivary glands respiratory tract, and genito urinary tract

The photoreceptor cells of the retinal rods which function in dim light contain visual purple or rhodopsin Visual purple is a compound of vitamin A with a protein The compound breaks down under illumination to visual yellow, which is composed of protein plus a yellow pigment, retinene Visual yellow can either reform visual purple or be converted to vitamin A and a protein by a rather complicated process involving cozymase, nicotinic acid, and vitamin E A large part of the liberated vitamin A is used again, but the process is not entirely reversible, and a supply of vitamin A is required for replacement The visual purple normally regenerates in a few *minutes of darkness or dim light* This is the basis of the phenomenon of *dark adaptation* which is delayed or absent in subjects deficient in the vitamins A

Avitaminosis A The commonest manifestation of early or moderate vitamin A deficiency in man is *night blindness* This is a rather extreme term for what is usually poor adaptation to dim light Often there is no complaint on the part of the patient or else certain difficulty may be noted in doing close work

there is difficulty in vision

illuminated area for one which is relatively [illegible]

struments have been devised for the measurement of dark adaptation and are useful in the diagnosis of moderate degrees of vitamin A deficiency In using such instruments it should be recalled that conditions other than lack of vitamin A can lead to poor dark adaptation Xerophthalmia is a much less common condition than night blindness in the human although easily produced in experimental animals It is characterized by decreased production of tears and a dry *conjunctivitis* In advanced cases there is

no formation of tears whatever, and the conjunctiva becomes hard, brittle, and scaly At this stage bacterial infection and mechanical damage are likely to occur, possibly resulting in complete functional loss of vision and extensive anatomical damage to the eye In serious deficiencies similar changes can be detected in the epithelia of the respiratory tract, and of the genito urinary tract Even the skin may undergo excessive drying and cornification

Hypervitaminosis A Rather considerable excess of vitamin A may be taken for a considerable time without causing difficulty Overloading of the liver with vitamin A has been reported as producing hepatic damage, which in turn has produced an elevation of the blood alkaline phosphatase Possibly as a result of the excessive phosphatase, formation of new bone under the periosteum is observed

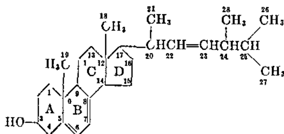
Human requirement A very serious effort has been made to determine the vitamin A requirements of the normal human subject (14) The subjects for this experiment consisted of 20 male conscientious objectors and 3 woman volunteers They were maintained on a diet from which were excluded such foods as dairy products liver and other organ meats all fats all fish and all green and yellow vegetables and fruits This diet was very low in vitamin A potency by biological assay with rats Chemical analysis for carotenes demonstrated a maximum of 70 international units The normal content of carotenes in the plasma of these subjects fell rather rapidly and approached zero levels during the first few weeks on the experimental diet The vitamin A of the plasma however fell much more slowly In one subject there was no decline in plasma vitamin A for 22 months Taking the subjects as a group a mean value of 88 international units per 100 ml of plasma during the first two months of the experiment fell to 74 international units in 9 months and in a reduced number of subjects was still 61 international units after 14 months The response of these subjects to tests of dark adaptation demonstrated a greater relationship to the season of the year than to vitamin A depletion In only three of the subjects could failure of dark adaptation be demonstrated as significantly different from that to be expected in relation to the season of the year These three subjects had 40 or less international units of vitamin A in the blood plasma In these three cases a daily dose of 1300 international units gradually improved dark adaptation and restored normal levels of plasma vitamin A The daily requirement for vitamin A, obtained as a result of this entire experiment, is set at 2500 international units of vitamin A per day the equivalent of which would be 4000 international units of beta carotene in oil, 7500 international units of carotene in cooked green vegetables, or 12 000 international units of carotene in the form of boiled carrots No symptoms of vitamin A deficiency appeared in these subjects

other than the 3 cases mentioned of unsatisfactory dark adaptation Some of the subjects continued the experiment for two years The conclusion was drawn that the human body mobilizes and expends the vitamin A reserves of the liver with notable economy during periods of vitamin A depletion There was no evidence of bacterial synthesis of carotenes to any effective degree although a number of micro-organisms including *Staphylococcus aureus* can perform this synthesis

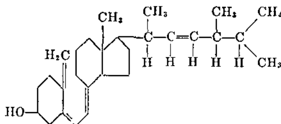
VITAMINS D

There are several substances which have a vitamin D action This action can be briefly summarized as the ability to protect the growing child or young animal against rickets Two of these substances occur in effective concentrations in a limited number of foods but their inactive precursors or provitamins are more widely distributed The conversion of provitamins D to the effective vitamins takes place in nature by the action of solar ultraviolet radiation Irradiation of *ergosterol* a sterol of plant origin yields vitamin D_2 Ergosterol was named from its first known source which was ergot a fungus growing on rye It is also a product of yeasts molds and other fungi Irradiation of ergosterol with either ultraviolet or cathode rays yields two intermediate products lumisterol and tachysterol prior to the formation of *calciferol* (vitamin D_2) The term vitamin D_1 now obsolete referred to a molecular compound of calciferol and lumisterol The formulas for ergosterol (formula XVIII) and for calciferol (formula XIX) are given omitting those of the intermediate products and those of the further products of irradiation isopyrocalciferol and pyrocalciferol toxisterol and suprasterols I and II Irradiation of *7 dehydrocholesterol* (formula XX) an animal sterol yields vitamin D_3 (formula XXI) which is the form of vitamin D characteristic of cod liver oil Other fish oils may contain vitamin D_2 in considerable amounts along with vitamin D_3 The chemical name of vitamin D_3 dimethyldihydrocalciferol is seldom used Both vitamins D_2 and D_3 are available in crystalline form and in solution for therapeutic and prophylactic uses

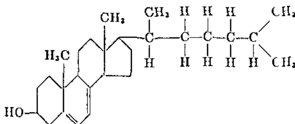
The higher plants contain insignificant amounts of vitamins D or none at all Yeasts and molds contain abundant provitamin D_2 which becomes activated in sunlight which probably explains the vitamin content observed in some plant products such as cocoa shells and hay Most animal tissues likewise contain little vitamin D but there are certain striking exceptions to this statement the livers and other viscera of many species of fish as well as the fat of these fish the fats of fish eating animals the eggs of all birds and the milks of all milk yielding animals all contain vitamin D chiefly in the form of D_3 Fish oils vary widely in vitamin D content the liver oil of the bluefin tuna contains 40 000 international units



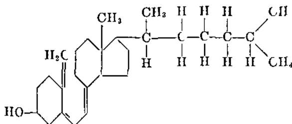
XVIII Ergosterol (provitamin D_2)



XIX Calciferol (vitamin D_2)



XX 7-Dehydrocholesterol (provitamin D_3)



XXI Vitamin D_3

per gram of halibut and mackerel about 1000, of cod about 100, of sturgeon none. The international unit is the vitamin D activity of 0.025 micrograms of pure calciferol, an amount which serves also as the U. S. P. and

A O A C unit The vitamin D content of eggs varies with the diet of the bird Milks human and bovine, usually contain less than 40 units to the quart Nearly all brands of evaporated milk are fortified with vitamin D 400 units to the reconstituted quart Many fresh milks are similarly fortified 400 units to the quart The provitamins D are much more abundantly distributed than the vitamins Land animals in general form their own vitamins D from provitamins which are most concentrated in the skin where they may be converted by sunlight The manner of synthesis of the provitamins by organisms is not fully known The known facts about the synthesis of steroids in general are summarized in Chapter 13 Certain simple organisms as for example *Aspergillus niger*, can accomplish the total synthesis of ergosterol with sodium acetate as the sole source of carbon This is in line with the evidence for the synthesis of cholesterol in the animal from acetyl groups

Properties The vitamins D are soluble in fats and fat solvents insoluble in water They have a characteristic absorption spectrum in the ultraviolet which is used in methods of vitamin D analysis of fish oils and other rich sources There are also certain reactions with antimony trichloride and with aluminum chloride in the presence of pyrogallol which have been made the basis of quantitative analyses The vitamins D are stable at all temperatures commonly used in the preparation of foods even in the presence of air Rancidity developing in fats or oils impairs the vitamin D content

The bioassay of vitamin D is described in detail in the United States Pharmacopoeia In brief summary young rats depleted of vitamin D are fed various amounts of the test material while control rats on the same diet are fed standard cod liver oil There are several tests applied to the animals to demonstrate cure of rickets One of these, the line test utilizes the proximal end of the tibia or the distal end of the radius or ulna The bone is sectioned longitudinally and treated with 2 per cent silver nitrate for one minute which converts calcium phosphate to silver phosphate After washing and exposure to light calcified areas will show a black stain The indication of healing rickets is a line of new calcification through the junction of epiphysis and diaphysis If chicks are used for assay the ash content of the tibiae is measured Assays on different animal species are usually not in agreement By the rat test vitamins D_2 and D_3 have the same potency 40 000 000 international units per gram In chicks 35 units (as measured by rat assay) of D_2 must be given to produce the effect of one unit of D_3 In the prevention or cure of human rickets no difference has been shown between D_2 and D_3

Function It was indicated at the outset that vitamin D activity is concerned with the prevention and cure of rickets Rickets is osteomalacia

(see page 588) occurring in growing bone and can result from deficiency of calcium, phosphate, or most commonly from deficiency of vitamin D The deposition of bone salt requires obviously the presence of calcium and of phosphate in the diet, but with abundant amounts of these minerals present and deficiency of vitamin D, calcification will still not take place in a normal manner The orderly advance of capillaries into the cartilage, and the uniform degeneration of cartilage cells just before the capillaries reach them, are replaced by a confused and irregular advance of calcification, with islands of persisting cartilage cells Organic matrix of bone is laid down but it is incompletely calcified and can be distorted by weight-bearing or by the pull of muscles Administration of adequate vitamin D, even if the supply of calcium and of phosphate is minimal, will normalize this situation in the area where normal calcification would have been taking place if the deficiency had not occurred The abnormal region of irregular bone formation and delayed calcification is slowly repaired later The line of healing can be shown by the x ray on account of its greater content of bone salt than the adjacent regions Vitamin D is involved in calcification through its influence upon at least two portions of the process the absorption of calcium and phosphorus from the intestine, and the actual process of ossification as localized in the bone The chemical mechanism of its action in either of these locations is not known

The deformities produced while rickets is present may persist and be made permanent as calcification becomes normal The bones are stiffened in their deformed position as the abnormal area belatedly calcifies The location and nature of the deformities depend not only upon the severity of the deficiency but upon the age of the child at the time of the disease *Craniotabes* is characteristic of rickets at an early age (3 to 8 months) and consists of thinning and softening of the bones of the skull, or of limited areas of those bones The cranial sutures are abnormally wide and the affected bone areas indent easily with pressure, returning to their original shape by their inherent elasticity The permanent deformities likely to result from rickets active in the cranium are the "Olympian brow" and the square head " These occur from pressure upon the sides and back of the head exerted by the weight of the head itself as the child lies supine or on either side Deformities which occur at later ages, listed more or less in sequence, are those of the thorax such as Harrison's groove" marking the pull of the diaphragm upon its attachments to the ribs, and the ' rachitic rosary" consisting of enlargement of the ribs at the costochondral junctions, later come deformities of the spine, the pelvis, and the lower extremities

The deformities briefly noted above are preceded by definite biochemical changes, of which the earliest is the increase in plasma alkaline phosphatase (see page 585) above the normal infantile limits of 3 to 12 Bodansky units

This change is also the most persistent of the biochemical abnormalities and lasts well into the stage of healing, denoting increased osteoblastic activity Plasma *inorganic phosphate* is characteristically decreased in human rickets, in this respect note that infants and children have normally higher values for inorganic phosphate than the adult No normal baby will *have an inorganic phosphate level below 5 mgm per 100 ml* plasma and this level may run as high as 7 mgm , the normal range for adults is 3 to 4 5 mgm , and the values for older children are intermediate Values of inorganic phosphate in infant plasma below 4 mgm per 100 ml are practically diagnostic for rickets In severe cases they may fall below half this value The calcium of the blood serum frequently remains normal in rickets and when there is a depressed value for calcium, tetany may be a complication (see page 588) These values refer only to human rickets in its *usual form—rickets from vitamin D deficiency They do not apply to* rickets experimentally produced in animals, nor to those rare cases of human rickets resulting from calcium or phosphate deficiency in the presence of adequate vitamin D, nor to
other organic disease or to extreme
rickets can not occur in the adult, s
salt deposition in growing bone Rickets can occur in the unborn or new born child, but the most susceptible age is the middle of the first year of life Other diseases can produce similar skeletal deformities, hence the value of biochemical diagnostic aids and of x ray studies

Requirements Rickets does not exist where culture and climate permit mothers and infants to benefit by reasonable exposure to unfiltered sunlight The infant who gets no exposure to sunlight requires 400 units a day of vitamin D_2 or D_3 With *smaller amounts, less of the* calcium of the diet is retained and skeletal growth is slower, with larger amounts up to around 1800 units a day there is no further increase in growth or percentage of calcium retained If amounts larger than 1800 units a day are given over long periods of time there is an adverse effect upon appetite and general growth These facts have been established by study of large groups of infants under well controlled conditions The requirements of older children are less definitely demonstrable by controlled experimental study, but it is well known that rickets can occur as long as growth continues In such studies as have been done, it appears *that throughout the* growth period the same intake of 400 units per day permits optimal utilization of calcium It should be kept in mind that the requirements of calcium and phosphate are greater in older children and in adolescents, and *that vitamin D will not compensate* for mineral deficiencies Once full growth has been achieved, no need for vitamin D beyond the minimal amounts gained from the normal diet and from normal exposure to sun

light has been demonstrated It is considered advisable for those who are on restricted diets and who lead an indoor life to take a small supplementary ration of vitamin D, but such advice is common sense precaution rather than scientifically established necessity Causes other than vitamin D deficiency appear to explain adequately the calcium losses from the bones of elderly people, and simple vitamin D therapy is not commonly successful in such cases The one situation in adult life where vitamin D becomes essential is during the latter half of pregnancy and the following period of lactation It is probable that 400 units per day is adequate here, but many medical authorities have advised the use of 800 units Even if the mother is receiving such a supplement, the breast fed baby should be given the usual supplementary vitamin D, since the output of vitamin D in the milk is variable and not fully reliable

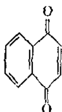
The treatment of actual rickets involves more than the supply of adequate intakes of calcium phosphate, and vitamin D, although these are of course essential Rickets will heal if the normal requirements of these substances are provided, but healing will be more rapid if larger doses of vitamin D, from 1000 to 4000 units daily, are used Still larger doses are used under special circumstances, for discussion of which the textbooks and literature of pediatrics should be consulted With 1000 units a day, plasma phosphate will reach normal in about 10 days, and evidence of incipient healing can be demonstrated by x ray in about 20 days

Toxicity of vitamin D must be kept in mind whenever large doses of vitamin D are considered for infants or children, or whenever administration of supplementary vitamin D is considered for the adult It has already been noted that doses of over 1800 units a day may if long continued, produce loss of appetite and slowing of growth in infants Much larger doses have been administered to adults daily for supposed therapeutic effects in various diseases, particularly of the skin and the joints In some such patients calcification of bursae has occurred also deposition of calcium salts in kidneys, heart bronchi, and blood vessels with serious disturbances of function There have been a few fatalities, both in infants and adults attributed to overdosage of the vitamins D, the immediate cause of death was failure of kidney function

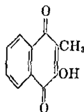
VITAMINS K

The naturally occurring vitamins K_1 and K_2 are derivatives of 1,4-naphthoquinone (formula XXII) which itself possesses moderate antihemorrhagic activity, as does phthiocol (formula XXIII) from the tubercle bacillus In the natural vitamins K, the hydroxyl group of phthiocol is substituted by a long side chain which is phytyl in vitamin K_1 (formula XXIV) and difarnesyl in vitamin K_2 (formula XXV) Vitamin K_1 occurs

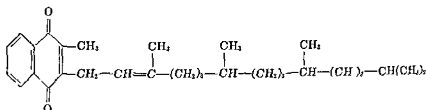
chiefly in the green leaves of plants K_2 in micro organisms especia bacteria The latter is the form produced by bacterial action in the testines of men and animals In the above substances convers on of t quinone to the corresponding hydroquinone brings about no loss of activit Any substitution in the benzene (not the quinone) ring causes comple loss of activity The 2 methyl group is necessary for a high level of activit



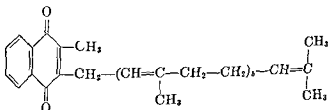
XXII 1 4 Naphthoquinone



XXIII Phthiocol



XXIV Vitamin K_1



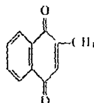
XXV Vitamin K_2

The substituent on the 3 position can be phytyl or difarnesyl as in the natural vitamins or a simpler aliphatic chain or a hydroxyl group as in phthiocol with little alteration in vitamin activity If the 3 position is unsubstituted we have a synthetic vitamin K 2 methyl 1 4 naphtho quinone or menadione (formula XXVI) which is 2 to 4 times more active than natural vitamin K_1 One microgram of synthetic vitamin K con stitutes one A O A C unit no international nor U S P unit has been adopted

One or another of the vitamins K will function to catalyze the formation of prothrombin in the liver Deficiency of the vitamins K leads to deficient

circulating prothrombin and consequent prolonged clotting time This defect can be remedied by administration of a vitamin K, provided the liver is not too diseased to produce prothrombin The K vitamins have no coagulant action *in vitro* Since natural vitamin K_1 is present in most normal diets and since vitamin K_2 is produced by the action of intestinal bacteria deficiency of K vitamins is uncommon in otherwise healthy people Deficiency may result from failure to absorb the vitamin as in disturbances of lipid absorption such as in sprue or protracted dysentery or in the absence of bile from the intestine as in obstructive jaundice Protracted use of antibacterial drugs, such as sulfonamides, may dangerously cut down the bacterial population of the intestine and thus cause production of vitamin K_2 to fail Much less commonly the deficiency may result from lack of adequate food, or bluntly, starvation

Each newborn infant starts extra uterine life with no intestinal bacteria

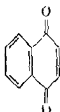


XXVI Menadione

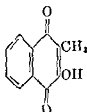
This situation is spontaneously remedied but several days may be required for vitamin K_2 production to get well under way in the intestine *Hemorrhagic disease of the newborn* results from vitamin K deficiency, and appears characteristically on the second or third day If death does not occur from loss of blood or intracranial hemorrhage, recovery occurs within a few days as vitamin K_2 is produced by the increasing bacterial flora of the intestine This disease can be prevented by small doses of vitamin K—for example one mgm of the synthetic product—given to the mother weekly during the final few weeks of pregnancy and daily when labor is expected to start The newborn infant may be given 0 5 mgm at birth and one mgm daily in divided doses for the critical 3 days Water soluble products such as synthetic vitamin K combined with $NaHSO_3$ may be given orally to infants

Whatever the cause, deficiency of the vitamins K is manifested by low prothrombin levels in the blood, prolonged clotting time, and hemorrhage The hemorrhage does not occur without previous injury, but the injury may be so slight as to be trivial were it not for the delay in blood clotting Prothrombin may be estimated in the blood by measurement of the clotting

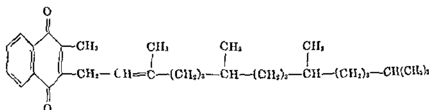
chiefly in the green leaves of plants, K_2 in micro organisms especially bacteria. The latter is the form produced by bacterial action in the intestines of men and animals. In the above substances, conversion of the quinone to the corresponding hydroquinone brings about no loss of activity. Any substitution in the benzene (not the quinone) ring causes complete loss of activity. The 2 methyl group is necessary for a high level of activity.



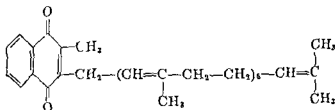
XXII 1,4 Naphthoquinone



XXIII Phthiocol



XXIV Vitamin K_1



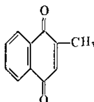
XXV Vitamin K_2

The substituent on the 3 position can be phytyl or difarnesyl as in the natural vitamins, or a simpler aliphatic chain, or a hydroxyl group, as in phthiocol, with little alteration in vitamin activity. If the 3 position is unsubstituted we have a synthetic vitamin K, 2-methyl 1 4 naphthoquinone, or menadione (formula XXVI), which is 2 to 4 times more active than natural vitamin K_1. One microgram of synthetic vitamin K constitutes one A O A C unit, no international nor U S P unit has been adopted.

One or another of the vitamins K will function to catalyze the formation of prothrombin in the liver. Deficiency of the vitamins K leads to deficient

circulating prothrombin, and consequent prolonged clotting time This defect can be remedied by administration of a vitamin K, provided the liver is not too diseased to produce prothrombin The K vitamins have no coagulant action *in vitro* Since natural vitamin K_1 is present in most normal diets and since vitamin K_2 is produced by the action of intestinal bacteria, deficiency of K vitamins is uncommon in otherwise healthy people Deficiency may result from failure to absorb the vitamin as in disturbances of lipid absorption such as in sprue or protracted dysentery, or in the absence of bile from the intestine as in obstructive jaundice Protracted use of antibacterial drugs, such as sulfonamides, may dangerously cut down the bacterial population of the intestine and thus cause production of vitamin K_2 to fail Much less commonly the deficiency may result from lack of adequate food, or bluntly, starvation

Each newborn infant starts extra uterine life with no intestinal bacteria



XXVI Menadione

This situation is spontaneously remedied but several days may be required for vitamin K_2 production to get well under way in the intestine *Hemorrhagic disease of the newborn* results from vitamin K deficiency, and appears characteristically on the second or third day If death does not occur from loss of blood or intracranial hemorrhage, recovery occurs within a few days as vitamin K_2 is produced by the increasing bacterial flora of the intestine This disease can be prevented by small doses of vitamin K—for example, one mgm of the synthetic product—given to the mother weekly during the final few weeks of pregnancy and daily when labor is expected to start The newborn infant may be given 0 5 mgm at birth and one mgm daily in divided doses for the critical 3 days Water-soluble products, such as synthetic vitamin K combined with $NaHSO_3$ may be given orally to infants

Whatever the cause, deficiency of the vitamins K is manifested by low prothrombin levels in the blood, prolonged clotting time, and hemorrhage The hemorrhage does not occur without previous injury, but the injury may be so slight as to be trivial were it not for the delay in blood clotting Prothrombin may be estimated in the blood by measurement of the clotting

chiefly in the green leaves of plants, K_2 in micro organisms, especially bacteria The latter is the form produced by bacterial action in the intestines of men and animals In the above substances, conversion of the quinone to the corresponding hydroquinone brings about no loss of activity Any substitution in the benzene (not the quinone) ring causes complete loss of activity The 2 methyl group is necessary for a high level of activity

XXII 1,4 Naphthoquinone

XXIII *Phthiocol*

$-CH_2-CH=C(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)_2$

XXIV Vitamin K_1

$-CH_2-(CH=C(CH_3)-CH_2-CH_2)_5-CH=C(CH_3)_2$

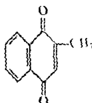
XXV Vitamin K_2

The substituent on the 3 position can be phytyl or difarnesyl, as in the natural vitamins, or a simpler aliphatic chain, or a hydroxyl group, as in phthiocol, with little alteration in vitamin activity If the 3-position is unsubstituted, we have a synthetic vitamin K, 2 methyl 1,4 naphthoquinone, or menadione (formula XXVI), which is 2 to 4 times more active than natural vitamin K_1 *One microgram of synthetic vitamin K constitutes one* A O A C unit, no international nor U S P unit has been adopted

One or another of the vitamins K will function to catalyze the formation of prothrombin in the liver Deficiency of the vitamins K leads to deficient

circulating prothrombin, and consequent prolonged clotting time This defect can be remedied by administration of a vitamin K provided the liver is not too diseased to produce prothrombin The K vitamins have no coagulant action *in vitro* Since natural vitamin K_1 is present in most normal diets and since vitamin K_2 is produced by the action of intestinal bacteria, deficiency of K vitamins is uncommon in otherwise healthy people Deficiency may result from failure to absorb the vitamin as in disturbances of lipid absorption such as in sprue or protracted dysentery or in the absence of bile from the intestine as in obstructive jaundice Protracted use of antibacterial drugs such as sulfonamides, may dangerously cut down the bacterial population of the intestine and thus cause production of vitamin K_2 to fail Much less commonly the deficiency may result from lack of adequate food or bluntly, starvation

Each newborn infant starts extra uterine life with no intestinal bacteria



XXVI Menadione

This situation is spontaneously remedied but several days may be required for vitamin K_2 production to get well under way in the intestine *Hemorrhagic disease of the newborn* results from vitamin K deficiency, and appears characteristically on the second or third day If death does not occur from loss of blood or intracranial hemorrhage recovery occurs within a few days as vitamin K_2 is produced by the increasing bacterial flora of the intestine This disease can be prevented by small doses of vitamin K—for example one mgm of the synthetic product—given to the mother weekly during the final few weeks of pregnancy and daily when labor is expected to start The newborn infant may be given 0 5 mgm at birth and one mgm daily in divided doses for the critical 3 days Water soluble products such as synthetic vitamin K combined with $NaHSO_3$ may be given orally to infants

Whatever the cause deficiency of the vitamins K is manifested by low prothrombin levels *in the blood prolonged clotting time,* and hemorrhage The hemorrhage does not occur without previous injury, but the injury may be so slight as to be trivial were it not for the delay in blood clotting Prothrombin may be estimated in the blood by measurement of the clotting

chiefly in the green leaves of plants, K_2 in micro organisms, especially bacteria The latter is the form produced by bacterial action in the intestines of men and animals In the above substances, conversion of the quinone to the corresponding hydroquinone brings about no loss of activity Any substitution in the benzene (not the quinone) ring causes complete loss of activity The 2 methyl group is necessary for a high level of activity

XXII 1,4 Naphthoquinone

XXIII Phthiocol

XXIV Vitamin K_1

XXV Vitamin K_2

The substituent on the 3 position can be phytyl or difarnesyl, as in the natural vitamins, or a simpler aliphatic chain, or a hydroxyl group, as in phthiocol, with little alteration in vitamin activity If the 3 position is unsubstituted we have a synthetic vitamin K, 2 methyl 1,4 naphthoquinone or menadione (formula XXVI), which is 2 to 4 times more active than natural vitamin K_1 One microgram of synthetic vitamin K constitutes one A O A C unit, no international nor U S P unit has been adopted

One or another of the vitamins K will function to catalyze the formation of prothrombin in the liver Deficiency of the vitamins K leads to deficient

soluble in water, and stable to the heat involved in cooking They are converted by oxidizing agents to inactive quinones, but are not oxidized under ordinary conditions of food preparation They are also destroyed, as far as vitamin effect is concerned, by ultraviolet radiation

The tocopherols occur chiefly in plants, especially in certain vegetable oils including the oils of wheat germ, cottonseed, and rice germ There is no vitamin E in olive oil, very little in peanut oil Other sources include lettuce and alfalfa, where the vitamin is present in the lipids of the leaves Small amounts are present in foods of animal origin particularly beef liver, but there is no evidence of synthesis of vitamins E in the animal Fats and oils containing tocopherols develop rancidity by oxidation more slowly than those which contain no vitamin E or other *antioxidant* Other antioxidants include certain phenols, naphthols and quinones Tocopherol, synthetically produced from alkylated hydroquinone and phytyl halide, is often added to fats and foodstuffs to delay spoilage by oxidative rancidity It will be recalled that vitamin A and the carotenes are susceptible to oxidative destruction They can be stabilized *in vitro* by tocopherols, and such stabilization may be a function of vitamin E in the gastrointestinal

photometric analysis is highly satisfactory in pure or nearly pure solutions utilizing an absorption maximum in the ultraviolet at 294 millimicrons Biological assay may be based upon the prevention of testicular atrophy in the rat (12)

Bulls cocks, and male rats and mice depleted of vitamin E by a deficient diet show a decrease in motility of spermatozoa, an increase in the proportion of abnormally formed spermatozoa and eventually degeneration of the germinal epithelium The last effect is not reversible by vitamin E administration while the earlier effects may be so normalized In female

of vitamin E With further prolongation of deficiency irreversible uterine degeneration occurs Hens deficient in vitamin E produce eggs in which the embryo chicks die before reaching the hatching stage In animals of both sexes, degeneration of nerves and striated muscles has been observed in severe deficiencies, associated with creatinuria Affected tissues first show increased oxygen consumption, but later degenerate and are replaced by scar tissue In such deficient animals, transamination is diminished up to 50 per cent

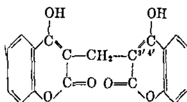
soluble in water, and stable to the heat involved in cooking They are converted by oxidizing agents to inactive quinones but are not oxidized under ordinary conditions of food preparation They are also destroyed, as far as vitamin effect is concerned, by ultraviolet radiation

The tocopherols occur chiefly in plants especially in certain vegetable oils including the oils of wheat germ, cottonseed and rice germ There is no vitamin E in olive oil very little in peanut oil Other sources include lettuce and alfalfa, where the vitamin is present in the lipids of the leaves Small amounts are present in foods of animal origin particularly beef liver, but there is no evidence of synthesis of vitamins E in the animal Fats and oils containing tocopherols develop rancidity by oxidation more slowly than those which contain no vitamin E or other *antioxidant* Other antioxidants include certain phenols naphthols and quinones Tocopherol, synthetically produced from alkylated hydroquinone and phytyl halide, is often added to fats and foodstuffs to delay spoilage by oxidative rancidity It will be recalled that vitamin A and the carotenes are susceptible to oxidative destruction They can be stabilized *in vitro* by tocopherols and such stabilization may be a function of vitamin E in the gastrointestinal tract and in tissues Several chemical tests have been devised for the determination of vitamin E but none of these has had wide application in human physiology or medicine for reasons which will appear later Spectrophotometric analysis is highly satisfactory in pure or nearly pure solutions, utilizing an absorption maximum in the ultraviolet at 294 millimicrons Biological assay may be based upon the prevention of testicular atrophy in the rat (12)

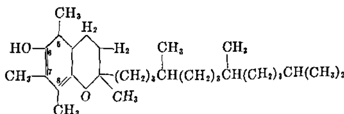
Bulls cocks and male rats and mice depleted of vitamin E by a deficient diet show a decrease in motility of spermatozoa, an increase in the proportion of abnormally formed spermatozoa and eventually degeneration of the germinal epithelium The last effect is not reversible by vitamin E administration while the earlier effects may be so normalized In female rats made deficient in vitamin E there occurs progressively as the deficiency continues (a) prolongation of gestation (b) stillbirths, and (c) death and resorption of the unborn young These effects cease with administration of vitamin E With further prolongation of deficiency irreversible uterine degeneration occurs Hens deficient in vitamin E produce eggs in which the embryo chicks die before reaching the hatching stage In animals of both sexes degeneration of nerves and striated muscles has been e in severe deficiencies associated with creatinuria Affected show *increased oxygen consumption but later degenerate* by scar tissue In such deficient animals transamination to 50 per cent

time in the presence of excess thromboplastin, by techniques described in detail in manuals of clinical pathology

Vitamin K deficiency caused by exclusion of bile from the intestine can be treated by oral administration of bile salts with additional vitamin K Other disturbances of vitamin K absorption call for parenteral administration of vitamin K which usually shows a favorable response within 6 hours The daily requirement has not been established, but a few milligrams is adequate for the correction of ordinary deficiencies A specific antagonist of vitamin K, *dicoumarol* (formula XXVII), inhibits production *of prothrombin and large doses of vitamin K of the order of 40 mgm* are required to overcome such inhibition Vitamins K_1 and K_2 are non toxic



XXVII Dicoumarol [3,3'-Methylene bis(4 hydroxycoumarin)]



XXVIII alpha Tocopherol

Menadione has caused vomiting, albuminuria, and porphyrinuria in doses of 180 mgm or more Such large dosage serves no useful therapeutic purpose

TOCOPHEROLS

A physiological action observed in experimental animals but not in man and designated as that of "vitamin E" is characteristic of three known compounds, all derivatives of chromane The best known and most potent is alpha tocopherol *(formula XXVIII) The international* unit of vitamin E activity is that of 1 mgm of synthetic alpha tocopherol acetate Beta tocopherol lacks the methyl group on carbon 7, gamma tocopherol lacks the methyl group on carbon 5 and is isomeric with beta tocopherol Alpha tocopherol has twice the potency of beta or gamma tocopherol These three tocopherols are all soluble in fats and fat *solvents, very* sparingly

soluble in water, and stable to the heat involved in cooking They are converted by oxidizing agents to inactive quinones, but are not oxidized under ordinary conditions of food preparation They are also destroyed, as far as vitamin effect is concerned, by ultraviolet radiation

The tocopherols occur chiefly in plants especially in certain vegetable oils, including the oils of wheat germ, cottonseed, and rice germ There is no vitamin E in olive oil very little in peanut oil Other sources include lettuce and alfalfa, where the vitamin is present in the lipids of the leaves Small amounts are present in foods of animal origin, particularly beef liver, but there is no evidence of synthesis of vitamins E in the animal Fats and oils containing tocopherols develop rancidity by oxidation more slowly than those which contain no vitamin E or other *antioxidant* Other antioxidants include certain phenols, naphthols, and quinones Tocopherol, synthetically produced from alkylated hydroquinone and phytyl halide, is often added to fats and foodstuffs to delay spoilage by oxidative rancidity It will be recalled that vitamin A and the carotenes are susceptible to oxidative destruction They can be stabilized *in vitro* by tocopherols, *and such stabilization may be a function of vitamin E in the gastrointestinal* tract and in tissues Several chemical tests have been devised for the determination of vitamin E but none of these has had wide application in human physiology or medicine for reasons which will appear later Spectrophotometric analysis is highly satisfactory in pure or nearly pure solutions, utilizing an absorption maximum in the ultraviolet at 294 millimicrons Biological assay may be based upon the prevention of testicular atrophy in the rat (12)

Bulls, cocks, and male rats and mice depleted of vitamin E by a deficient diet show a decrease in motility of spermatozoa an increase in the proportion of abnormally formed spermatozoa and eventually degeneration of the germinal epithelium The last effect is not reversible by vitamin E administration while the earlier effects may be so normalized In female rats made deficient in vitamin E there occurs progressively as the deficiency continues (a) prolongation of gestation, (b) stillbirths, and (c) death and resorption of the unborn young These effects cease with administration of vitamin E With further prolongation of deficiency irreversible uterine degeneration occurs Hens deficient in vitamin E produce eggs in which the embryo chicks die before reaching the hatching stage In animals of both sexes degeneration of nerves and striated muscles has been observed in severe deficiencies associated with creatinuria Affected tissues first show increased oxygen consumption but later degenerate and are replaced by scar tissue In such deficient animals, transamination is diminished up to 50 per cent

Although sterility and abortion in cattle and hogs have been in some instances overcome by the use of vitamin E, its application in the treatment of human infertility has led to no clear cut favorable results It is unlikely that such deficiency plays any significant part as a cause of sterility in men or women Neither has vitamin E been notably effective in the treatment of muscular dystrophies in the human The best therapeutic successes have been in *primary fibrositis, a disease characterized by fibrillar nodules* near joints, and by creatinuria The vitamins E are nontoxic No human requirement can be stated, since no signs or symptoms definitely related to tocopherol deficiency have been observed in man Human blood plasma contains one mgm or less of vitamins E per 100 ml, and the plasma level in man does not correlate with the dietary intake

VITAMIN U

This fat-soluble substance, of unknown chemical structure protects guinea pigs against the development of peptic ulcer following injections of histamine (3) The most reliable source of this anti ulcer substance is cabbage, numerous other vegetables egg yolk, and raw milk are less potent sources No connection has been established between deficiency of vitamin U and peptic ulcer in the human This vitamin is probably identical with the 'anti gizzard erosion factor" which is necessary in the diet of chicks

REFERENCES

1 Burch H B *et al* Fluorometric measurements of riboflavin and its natural derivatives in small quantities of blood serum and cells J Biol Chem 175 457–470 1948

2 Castle W B Observations on etiologic relationship of achylia gastrica to pernicious anemia I Effect of administration to patients with pernicious anemia of contents of normal human stomach recovered after ingestion of beef muscle Am J M Sc 178 748–764 1929

3 Cheney, G The nature of the antipeptic ulcer dietary factor Stanford M Bull 8 144 161 1950

4

5

pany 1951

6 Crandon J H *et al* Experimental human scurvy New England J Med 223 353–367 1940

7 Darby W J Advances in the therapy of nutritional deficiencies Am J Trop Med 31 183 191 1951

8 Faulkner J M and Taylor F H L Vitamin C and infection Ann Int Med 10 1867–1873 1937

9 Figueroa W F *et al* Lack of avitaminosis among chronic alcoholics Its rela

tion to fortification of cereal products and the general nutriture of the population J Clin Investigation 29 812 1950
10 FITZPATRICK J AND TOMPSETT S L The daily excretion of riboflavin, biotin pantothenic acid and nicotinic acid derivatives by normals J Clin Path 3 69-71 1950
11 HALEY T J AND FLESHER A M A toxicity study of thiamine hydrochloride Science 104 567-568 1946
12 HERRAIZ M L AND RADICE J C The biological assay of vitamin E by the male rat test ' Ann New York Acad Sc 52 68-93 1949
13 HORWITT M K et al Correlation of urinary excretion of riboflavin with dietary intake and symptoms of ariboflavinosis J Nutrition, 41 247 264 1950
14 HUME E AND KREBS H A Vitamin A requirements of human adults Medical Research Council Special Report Series No 264 London His Majesty s Stat Off 1949
15 JOLLIFFE N et al Clinical Nutrition New York Paul B Hoeber, Inc 1950
16 KIRK F AND CHIEFFI M Vitamin studies in middle aged and old individuals III Thiamine and pyruvic acid blood concentrations J Nutrition 38 353-360 1949
17 LAMPEN J O AND JONES M J The antagonism of sulfonamides by pteroylglutamic acid and related compounds J Biol Chem 164 485-486 1946
18 LEVINE S Z et al Effect of ACTH and some adrenocortical steroids on the metabolism of tyrosine and phenylalanine in premature infants Science 113 311-312 1951
19 LEVITAN B A The biochemistry and clinical application of vitamin P New England J Med 241 780-789 1949
20 MEIKLEJOHN A P AND PASSMORE R Nutrition and nutritional disease Ann Rev Med 2 129-154 1951
21 MELNICK D AND FIELD H JR Chemical determination of vitamin B_1 I Reaction between thiamine in pure aqueous solution and diazotized p aminoacetophenone J Biol Chem 127 505-514 1939 II Method for estimation of the thiamine content of biological materials with the diazotized p aminoacetophenone reagent Ibid 515-530 III Quantitative enzymic conversion of cocarboxylase (thiamine pyrophosphate) to the free vitamin Ibid 531-540
22 MUEHRCKE R C AND KARK R M Negative effects of vitamin B_{12} on blood of healthy individuals with special reference to eosinophils Proc Soc Exper Biol & Med 77 144 145 1951
23 PENNY J R AND BALFOUR B M The effect of vitamin C on mucopolysaccharide production in wound healing J Path & Bact , 61 171 178 1949
24 ROBERTS E Z et al Effect of vitamin B_{12} on phosphorus metabolism of Lactobacillus leishmannii J Bact 58 709-710 1949
25 SARGENT F II Folic acid Pteroylglutamic acid and related substances New England J Med 237 667-672 703 707 1947
26 SARGENT F II AND SARGENT V W Season nutrition and pellagra New England J Med 242 447-453 507-514 1950
27 SAYERS G The adrenal cortex and homeostasis Physiol Rev 30 241-320 1950
28 SOBEL A E AND SNOW S D The estimation of serum vitamin A with activated glycerol dichlorohydrin J Biol Chem 171 617-632 1947
29 SYDENSTRICKER V P et al Observations on the egg white injury in man and its cure with a biotin concentrate J A M A 118 1199-1200 1942

30 THORNTON, N C Carbon dioxide storage XIV The influence of carbon dioxide, oxygen, and ethylene on the vitamin C content of ripening bananas Contrib Boyce Thompson Inst, 13. 201–220, 1943

31 WEISS, S, AND WILKINS, R W The nature of the cardiovascular disturbances in nutritional deficiency states (beriberi) Ann Int Med, 11: 104–148, 1937

32 WILLIAMS, R J, *et al* *The Biochemistry of B Vitamins* New York, Reinhold, 1950

33 WOLBACH, S B The pathologic changes resulting from vitamin deficiency J A M A, 108. 7–13, 1937

CHAPTER 20

Infection

At some early stage of evolution, possibly before plants and animals had become clearly differentiated from each other, one or more species must have made the discovery that a very convenient source of food consists of the tissues of other organisms In such tissues may be found materials, already pre formed, suitable as energy sources and for building one's own tissues

The whole of the animal kingdom depends upon eating plant life of some form or other directly or indirectly, and thus obtains the protein, fat, and carbohydrate which it needs Only the green plants and the autotrophic bacteria are able to synthesize these substances from inorganic materials and carbon dioxide alone

Among some of the species which had discovered the importance and desirability of eating other organisms as a source of food, the expedient of *parasitism* developed After all, it is not economical to destroy the source of your food material completely, although some micro organisms, perversely enough, do this to their hosts We believe, however, they are in the minority Instead, it is easier to live in or on the host, making use of the food supply which he provides directly or indirectly This is parasitism, of which infection is an example

However, the organisms which are thus forced to act as a host to parasites do not themselves remain idle in the evolutionary sense Organisms in general have provided themselves with some kind of membrane or sheath, partly to protect themselves against invasion by other organisms from the outside Other mechanisms, to be described in the next section, have also been developed We are not without our defenses, therefore, against invasion by foreign organisms, and it is the chemical nature of these defenses which concerns us here The invaders are generally classified either as animal parasites, or as bacteria or fungi (which are generally considered forms of plant life), or as viruses, which are smaller than either and apparently simpler, in some extreme cases consisting entirely of molecules of nucleo proteins

RESPONSE TO INVASION

The human body has a number of mechanisms which oppose infection The most important of these is phagocytosis, which means that leukocytes are mobilized and literally eat up the invading organisms This, however, is rather a concern of pathology or immunology, and in spite of its great importance we shall not consider it further here From the biochemical aspect we are interested in the chemical changes which take place in the body when it attempts to resist infection The most important and most readily recognized of these changes is the production of antibodies

Antibodies may be defined as the substances which appear in the blood plasma or tissues of an animal within a few days after the parenteral introduction, either naturally, as in disease, or artificially, into that animal of certain types of foreign substances Artificially introduced foreign substances may be pure proteins, tissues or body fluids, bacterial cells, blood cells or cellular extracts or fractions The particular property which distinguishes an antibody is that it reacts specifically with the material which was introduced Any material which will evoke the production of antibodies is called an *antigen* Antigens may be pure protein or polysaccharide in nature or they may be complex biological substances containing proteins or polysaccharides An animal who has received injections of an antigen and who has subsequently formed specific antibodies is said, by convention, to be *immunized* to that particular antigen, and the serum containing specific antibodies is called an *immune serum*

Anaphylactic shock may occur when certain animals (e g , the guinea pig) receive a second injection of antigen 10 to 12 days after "active sensitization" with the same antigen, or 1 to 2 days after "passive sensitization" with the specific antibody The second, or "shocking," dose must be considerably larger than the minute amounts needed for active sensitization, and is more effective if given suddenly by the intravenous route Such anaphylaxis is a manifestation of the union of antigen with antibody *in vivo* Sublethal shocking doses are followed by a long period—days or weeks—in which the animal is refractory to further shocks Uterine or intestinal strips of sensitized animals, suspended in Ringer's solution, contract when antigen is added to the bath Manifestations of anaphylaxis vary in different animal species The guinea pig is subject to asphyxia, resulting from bronchial constriction Dogs typically vomit and become paralyzed, their arterial pressure falls and the heart beat is accelerated These symptoms appear to result chiefly from vasodilatation Rabbits dying of anaphylaxis show constriction of the pulmonary arterioles, dilatation of the right heart, with acute heart failure The *Arthus phenomenon* is local death of tissue at the sight of injection of antigen into an immunized animal

Antibodies and Their Production

Antibodies are plasma proteins belonging to the class of gamma globulins. Indeed, it has never been clearly established that there are any gamma globulins which are not antibodies. On the other hand it has been impossible to prove that all the gamma globulins are antibodies. Some might conceivably be normal gamma globulins of unknown function, but since *the body has to resist a large number of different types of infection,* it therefore has constantly on hand a number of different antibodies in varying amounts. If we could measure and add up all the antibodies present, they might account for the whole of the gamma globulins. Since we can not in fact do this, the question remains rather academic.

In any case the properties of antibodies are not greatly different from those of gamma globulins in general. In man they have a molecular weight of about 160,000 and it has been calculated, for example, that the human antipneumococcus antibody has a short axis 3.7 millimicrons in length, and a longer axis of 33.8 millimicrons (17).

Specificity. An outstanding characteristic of the antibodies is their specificity, that is, they tend to combine primarily with the invading organism or substances derived from it. However, there are limitations to this specificity even though it is so sharp that it allows the distinction of different species of blood or even different types of blood within the same species (see page 385). Antibodies, although specifically directed toward one invading organism or one of its components, are also capable as a rule of reacting with chemically related compounds. Thus, the antibodies produced by injecting rabbits with the egg albumin from the hen will also react with the egg albumin from the duck. Such cross reactions are also observed with different species of pathogenic micro organisms.

The general course of events in an infection is something like this. First, bacteria or other foreign organisms enter through the skin or a mucous surface and probably next pass to a regional lymph node. There they proceed to reproduce. Phagocytosis removes them from the blood as they are liberated into it, unless the disease is very serious, in which case bacteremia, meaning presence of bacteria in the blood, results. The phagocytic cells can carry the foreign invaders to various parts of the body, including the reticulo-endothelial system. Antibodies are formed in the reticulo-endothelial system, and possibly in other parts of the body such as in the lymphocytes.

Antibodies do not at once appear in the circulation following exposure of an animal to an antigen, and the animal does not become at once immune. Instead there is a latent period, usually of the order of six days or somewhat longer. When antibodies do begin to appear in the blood, they combine with the antigens which caused their appearance and apparently make

them more attractive to the phagocytes, so that the foreign invaders present in the blood are more rapidly removed, and may be removed from localized infections also Also, antibodies may have the property of neutralizing toxic substances produced by the invaders, as for example the toxin of the diphtheria bacillus The two principal functions of antibodies in infection seem to be neutralization of toxicity and sensitization of the invaders to phagocytosis

Evidently gamma globulin molecules must be modified in a specific way in order to become antibodies which can combine firmly with special antigens We do not know the exact nature of this modification, but it is supposed that it takes the form of a correspondence of positive and negative charges, and possibly hydrogen bonding sites, which enable the antibody to present a localized patch which matches one or more characteristic patches (complexes of positive and negative charges and sites of potential hydrogen bond formation) on the antigen

The exact number of these specialized sites on a molecule of antibody has not been determined Some immunologists have written as if they considered that there were large numbers of such sites It is more likely that the number of combining sites of antibody is not greater than two or three Certain theories have been proposed in the past which would limit it to one (4) It should be obvious that a difference of only two or three localized combining sites on the surface of a protein molecule of the size of gamma globulin would not make a very great difference in its chemical behavior This is especially true if as has been proposed by Pauling (18), the difference in the antibody molecule is not due to a difference in the order in which the amino acids follow each other in the polypeptide chain, but simply a difference in the way in which the polypeptide chain is folded In this case the amino acid analysis of antibody would be expected to be the same as that of total gamma globulin, and up to date no differences have been found Pauling considers that it is necessary to suppose that antibodies as a rule have two combining groups one on each end of the molecule, and he considers this an integral part of his theory His theory of the formation of antibody could still hold even if antibodies were in general equipped with only one specific combining site

Increase of gamma globulin in infection Since antibodies are gamma globulins, one would expect to see a marked increase of these proteins during infections and this can usually be observed The gamma globulin increase is not always exactly equal to the amount of specific antibody production although it usually runs parallel to it Boyd (5) found that the antibody in rabbits amounted to 40 to 70 per cent of the increase in globulin Bjørneboe (3) was able however, to produce antibodies in rabbits to

such a degree that this accounted for all of the globulins present in their sera

Combination Between Antibody and Antigen

Antibodies formed in response to infection usually combine specifically with the invading organism or some portion of it It is believed by immuno chemists that they do this by purely chemical combination the mechanism of which is not particularly different from that of the combination of other chemical compounds The combination is as a rule a very firm one and it is difficult to split antibody and antigen apart after they have combined The heat of reaction between antibody and antigen is high—about 3300 Kcal per mol (6) For these reasons it seems likely that the main forces acting are the combination between positively and negatively charged groups, for example RNH_3^+ and $RCOO^-$ This is also supported by studies on artificial antigens into which charged and uncharged groups have been introduced and measurements of the rapidity of the reaction between anti bodies and antigens

Since antibodies show a considerable degree of specificity the reaction can not be as simple as the mere combination of one positive group in the antibody with one negative group in the antigen or conversely However, if we imagine that on the antigen there are numerous characteristic patterns of positively and negatively charged groups and that the antibody has in some way duplicated this pattern (but in reverse) then the spatial configu ration might easily differ from one antigen or one portion of the same anti gen to another in such a way as to allow a very high degree of specificity Hydrogen bonds are thought to be important in holding protein molecules together and may operate here

Second stage of antibody-antigen combination. After the antibody and antigen have combined a second stage which is generally slower, may be observed This may consist of the precipitation of certain soluble anti gens, lysis in case the antigen is an invading micro organism or a foreign red cell, or neutralization if the antigen is a toxin or a virus The phenome non of agglutination which consists of the aggregation into clumps of micro organisms or foreign red cells is one of the easiest to observe and has already been described under blood groups (see Chapter 4)

Theories differ as to the mechanism by which this second stage is effected The older theory supported by Bordet (4) many years ago was that the second stage was a non specific colloidal phenomenon due to the fact that the charges on the antigenic particles had been discharged by their com bination with antibodies A more recent theory proposed independently by Marrack (16) and by Heidelberger and Kendall (12) proposes that t

second stage is merely a continuation of the first and is equally specific. Experiments designed to test this have not always been conclusive, but in some cases specificity of the second stage has certainly been demonstrated. Table 44 shows this (1). There is probably truth in both theories.

Proportions in which antibody and antigen combine. Antibody and antigen may combine in multiple proportions, which is not surprising if at least one of them has more than one combining group and antigen at least does have, judging by the results of all analyses made to date. Anti

TABLE 44

Specificity of hemagglutination (1)

ANTIGEN	ANTIBODY	RESULT
Ag + Bc	anti A	Homogeneous clumps of ghost cells
Ag + Bc	anti B	Homogeneous clumps of normal cells
Ag + Bc	anti A + anti B	Homogeneous clumps of normal cells and homogeneous clumps of ghost cells

Ag = ghost (i.e. partially hemolyzed for identification) red blood cells of Blood Group A

Bc = intact red cells of Group B

TABLE 45

Molecular compounds of thyroglobulin and its antibody observed (12)

EXTREME ANTIBODY EXCESS	ANTIBODY EXCESS END OF EQUIVALENCE ZONE	ANTIGEN EXCESS END OF EQUIVALENCE ZONE	ZONE OF PARTIAL INHIBITION	SOLUBLE COMPOUNDS IN INHIBITION ZONE
$A_{40}T$	$A_{4}T$	$A_{10}T$	$A_{2}T$	AT

A symbolizes antibody. T symbolizes thyroglobulin.

body may have two or more combining groups, although the exact number is still not established. This means that the combination of antibody and antigen, although it really follows the stoichiometric rules of ordinary chemistry, may result in a very large number of compounds of different composition. This is shown in table 45.

If insufficient antibody is added to an antigen, no visible results will follow, and it is customary to say that the reaction has been inhibited by excess of antigen. Excess of antibody does not always have the same effect, [illegible]

how much antibody is added, a compound is formed which contains more

and more antibody, although free antibody may be found in the supernatant fluid after the antibody antigen compound has separated out

Neutralization

There is usually a certain combination of antibody and antigen which neutralizes the antigen This can be observed if the antigen is a toxin or an enzyme There is also a certain mixture of antibody and antigen or rather a certain ratio between the concentrations in which they are mixed which gives the most rapid reaction (7) In some cases, as with diphtheria antitoxin this region of most rapid reaction corresponds well to the region of neutralization In other cases it has been found to be different

Flocculation Tests for Syphilis

If the blood of patients infected with syphilis contains antibodies to the spirochete it might be possible to demonstrate them by a precipitation test using a suitable extract of the infecting organism which might contain proteins carbohydrates and possibly lipids However it does not seem that the infecting organism of syphilis has yet been successfully cultivated in the laboratory or at least no organism so cultivated has been shown to be the causative agent of the disease

Nevertheless in the early days of immunology it was discovered that *in vitro* tests for syphilis could be made The first extracts were made from human syphilitic organs and were thought to contain an antigen characteristic of the spirochete These were tested against with the blood of patients and when a positive reaction occurred it was considered that this was a sign that the patient had anti syphilis antibodies, and thus by inference was infected with *Treponema pallidum*

Later it was shown that extracts of normal organs would likewise react in this fashion and this first led to doubts as to the validity of the test as a specific test for syphilis However it was shown clinically that except in the case of a few diseases such as malaria and vaccinia a positive reaction was generally associated with a syphilitic infection Thus the test although based initially on a false assumption has proved to be of practical value The direct precipitation or as it is usually called flocculation test, which we have outlined is now used a good deal in the tests for syphilis but historically it was preceded by another test which depends upon the use of complement which we must now discuss

Complement

If serum containing antibody is heated to 56°C for half an hour its ability to produce bactericidal and hemolytic reactions is lost although it may still precipitate antigens and neutralize toxins The lytic antibody has

not been lost, however, and this can be demonstrated by actual measurement (say by micro Kjeldahl determinations) of the antibody which combines with known amounts of antigen It can also be shown that the addition of normal serum will restore the bactericidal or hemolytic power of the immune serum Evidently some thermolabile material was destroyed in the heating process, this substance is called complement Such substances are found in the normal serum of most species, the serum of the guinea pig is a particularly rich source

At first it was natural to suppose that complement was a single substance, but fairly early observations indicated that this was not true If electrolytes were removed by dialysis from complement containing serum so that the euglobulins were precipitated, it was found that neither the supernatant nor the precipitate had the power of restoring the lytic power to a previously heated lytic serum If, however, the precipitate and supernate were recombined and added, they did have this power It was therefore obvious that complement could be separated into at least two components Other experiments have shown that there are other components of the action of complement which may be removed by the absorbing power of yeast cells or by treatment of the serum with ammonia Some of these more recently discovered fractions of complement are much more resistant to the action of heat than the fraction which was originally identified It is customary to designate the four fractions of complement which are known at present as C'_1, C'_2, C'_3, and C'_4 The fraction now designated as C'_1 was originally called midpiece and C'_2 was at one time called endpiece C'_1, C'_2, and C'_4 have been obtained in pure form as well defined proteins, although the C'_2 and C'_4 activity may reside in one and the same molecule of protein, which seems to be a mucoglobulin C'_1 is a globulin with an isoelectric point of 5 2 to 5 4 C'_3 has not been obtained in pure form but seems to be possibly a phospholipid or a phosphoprotein It will be convenient to lump all of these components together under the traditional name of complement in the discussion that follows

Complement fixation. It was observed a long time ago that when antibody and antigen combine they use up complement in the process, and if the amount of complement in the mixture is suitably adjusted, the antibody antigen reaction will quantitatively remove the complement activity This is called *complement fixation* Actually the first test for syphilis devised depended upon complement fixation rather than the direct demonstration of the reaction of supposed syphilitic antibodies with supposed syphilitic antigens This test, known as the *Wassermann test*, is carried out in the following manner The patient's serum together with a definite amount of guinea pig complement is placed in a test tube with a lipid extract, usually from normal beef heart (Bordet showed that extracts of normal organs

worked as well as extracts of syphilitic organs) Other lipid substances such as cholesterol are sometimes added to increase the sensitivity of the test If the patient has syphilis the antibodies in his blood will react with this "antigen" and in the process complement will be used up This reaction is, under ordinary circumstances invisible Next there is added to the test tube a mixture of sheep cells with a rabbit antibody which has the power of hemolyzing sheep cells but which has been heated to deprive it of its complement activity so that alone it will not cause lysis

If, after these sheep cells combined with anti sheep antibodies (which are spoken of as sensitized sheep cells) are added, they are observed to be hemolyzed, it follows that complement was still present in the mixture of the patient's serum and the lipid 'antigen This indicates in turn, that there had been no reaction between the patient's serum and the lipid antigen, and the patient probably does not have syphilis If, on the other hand, lysis of the added cells does not occur, it indicates that complement has been used up in the preliminary reaction between the patient s serum and the lipid antigen Most such reactions are diagnostic of syphilis It will be seen that the test is an indirect one We add an indicator system—the sensitized sheep cells—to see whether or not one component of our system has been removed by a reaction which we otherwise would not be able to see

The direct precipitation reaction for syphilis already described was developed after the Wassermann test by workers who accidentally made the observation that under suitable conditions and with the addition of suitable adjuvants, it was possible to see directly the reaction between the antibody, or reagin as it is called, in syphilitic serum and the lipid antigen

For a long time the complement fixation test was preferred and is still used to some extent Many workers, however, have shifted to the more direct precipitation or flocculation test, and in many cases when a Wassermann reaction is referred to, what is actually meant is some modification of the original Sachs Georgi precipitation test (15) (10) (13)

Complement fixation has also been utilized in the diagnosis of other diseases and in the detection of small amounts of blood It has the advantage that it makes visible in a dramatic fashion—by the lysis or non lysis of red cells—a phenomenon which otherwise is not very noticeable and which involves fairly small amounts of material

TOXINS

Some micro-organisms produce actively poisonous substances Examples of this are the toxic protein produced by the diphtheria bacillus and that produced by *Clostridium botulinum* It is customary to divide bacterial toxins into two classes, exotoxins and endotoxins The exotoxins, typified by diphtheria toxin, are given off by the body of the bacterium during its

growth and will remain in the culture medium if the organism is removed The endotoxins appear to be *intracellular constituents of the* bacterial cell which are not freed during its lifetime Some of them are probably identical with the protein carbohydrate lipid complexes which have been called Boivin antigens

No reason is known why organisms produce toxins, because in many cases this seems to gain them no advantage, and in fact, if it brings about the death of the host this results sooner or later in the death of the invading micro organisms or their immediate descendants

The tubercle bacillus produces a soluble substance, *tuberculin*, which is not generally classed as a toxin because it seems to have no toxic effects on animals not previously exposed However, it does have toxic effect on animals which have or have had tuberculosis and also on some of their cells when they are tested *in vitro* Tuberculin, as isolated by growing the tubercle bacillus, contains three main components, a protein, a polysaccharide, and a nucleic acid Two soluble native proteins designated as A and B have been identified, they differ in electrophoretic mobilities and therefore chemically

Certain pathogenic bacteria contain a spreading factor which has been identified with the enzyme hyaluronidase (see page 130) which causes depolymerization of the hyaluronic acid which forms the ground substance of the skin and other tissues It has been claimed that the degree of invasive ness of bacteria is largely determined by the amount of spreading factors which are present Some workers, however, have denied that there is much relation

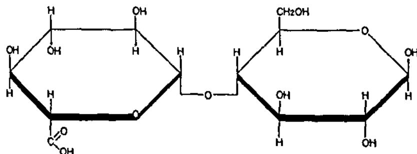
Although not toxins other components of micro organisms may have harmful effects during infection The polysaccharides of pneumococcus are non toxic, but since they combine with the special antibody which might otherwise cause the removal of the micro organisms from the blood and thus protect against this disease, they make it easier for the pneumococci to gain a foothold and carry on their invasion of the body

of glucose united by a glucoside link involving the aldehyde group of the glucuronic acid and carbon 4 of the glucose *This gives the compound* which is known as cellobiuronic acid (formula I) Some of the other pneumococcus polysaccharides also contain cellobiuronic acid but may contain glucose or other carbohydrates

IMMUNIZATION

Early attempts were made to use antibodies in the prevention and treatment of diseases One of the earliest successful applications involved the

production of diphtheria antitoxin by injecting horses with gradually increasing doses of diphtheria toxin They developed a neutralizing antibody in their serum and when such horse serum was given to children infected with diphtheria, it often had a very pronounced beneficial effect Similar neutralizing sera were developed for other toxins and antisera for bacteria such as the pneumococci were at one time in fairly wide use All of this has been changed by two main factors one is the gradual realization that it is on the whole better to have the patient manufacture his own antibodies in advance This can be done, for instance, by administering small doses of bacterial toxins or dead micro-organisms which will stimulate the antibody forming mechanism of the patient Absolute immunity is not always produced by this technique but it has been found reasonably successful in diseases such as typhoid, whooping cough, and tetanus It is not always necessary to inject an active toxin to obtain immunity to a



I Cellobiuronic acid

toxin It is often possible to detoxify the toxin without loss of its immunizing power by treatment with an agent such as formaldehyde

Another factor which has contributed very greatly to the decreasing use of animal sera in the treatment of disease has been the development of chemotherapeutic agents which enable the infection to be treated directly Although it does not always appear that these are successful with patients who can not produce at least some antibodies they will hold the growth of the invading organism in check until the patient has a chance to produce sufficient antibodies to counteract the infection

VIRUSES

The *viruses have certain characteristics in common* which *justify our* treating them as a group One of the most important is that they are capable of reproducing only inside of the cell of the susceptible host Other characteristics are the frequent presence in the infected cells of *inclusion bodies* the appearance of inflammation, mainly as a secondary phenomenon, and the proliferation or degeneration or both of the cells which are

affected At least two viruses are known which cause proliferation to the extent of producing tumors There are Shope's papilloma and Rous's sar coma (see Chapter 8)

Originally viruses were defined by their ability to go through pores in filters which would hold back bacteria, and were therefore called filtrable viruses and also by the fact that unlike bacteria they could not be seen under the microscope However, it is now known that they vary very much in size and that the vaccinia virus, for example, can be seen with microscopes using visible light Even the smaller virus particles can be seen with the aid of the electron microscope

Chemical Nature of Viruses

A study of the chemical nature of viruses has revealed that they vary a great deal in chemical composition One which has been studied extensively, although it is of no importance in human disease, is the tobacco mosaic virus which causes a characteristic disease of the tobacco plant This has been isolated in crystalline form The smallest particles which were ac tively infective have not been found to be smaller than about 15 x 280 millimicrons The tobacco mosaic virus appears to be composed entirely of protein and nucleic acid

It is not known exactly how the virus gets inside the cells of the host, which it must do in order to produce an infection, but it is known that many viruses have the power of attaching themselves strongly to the surface of various types of cells, including erythrocytes It is possible that this is the first stage in a process which later leads to a penetration of the cell and a proliferation of the virus inside of the cell The presence of nucleo protein in viruses and the fact that some viruses seem to consist almost entirely of nucleoprotein, has suggested that there may be some analogy between viruses and genes It will be noticed that both have the power of self-duplication inside of the cell and not outside of it

Immunity to Viruses

Another characteristic associated with virus diseases is that a single attack of the disease confers an immunity which often lasts for the lifetime of the individual However, there are a few notable exceptions to this The influenza virus confers a very short immunity which lasts only one to three years according to Burnet (8), and the common cold is notorious for conferring an extremely brief immunity, if indeed any at all However, these are exceptions which merely serve to point up the general rule, and all readers will be familiar with the immunity following virus diseases such as measles smallpox, and chicken pox

In the case of immunity to viruses, other factors in addition to anti bodies may be operating Although the evidence on this point is still con

troversial, one factor may be an increased resistance of the tissues themselves to the entry of the virus Also, there is some evidence that at least in some virus diseases, some living virus remains present in the body for a long time, possibly many years, after recovery The presence of this virus may stimulate the continuous production of sufficient antibody to keep the virus inactive and localized and to prevent reinfection

Immunization to viruses. It is much more difficult to grow viruses than to grow bacteria or even animal parasites, and it has been only in recent years that it has been possible to obtain sufficient amounts of non-infective purified virus to produce immunity by the injection of such material Other devices have been resorted to, therefore

One of the oldest devices is the introduction of the fully active virus by some unnatural portal of entry This is exemplified by the old practice of variolation, which was introduced into England from Turkey by Lady Mary Wortley Montagu Here the virulent smallpox virus was scratched directly into the skin instead of being allowed to enter the body through its natural route, the upper respiratory tract It was claimed that this produced a comparatively mild disease and recovery resulted in immunity

Such methods of producing antivirus immunity have never been really popular and are not likely to become so There is, in the first place, always the possibility that a typical or perhaps a fatal form of the disease may develop, and there is also the fact that the individual really has the disease and is therefore a danger to others, and might even be the starting point of an epidemic

Therefore, it would seem that what we need is a virus which has been deprived of its power of producing disease but still possesses its immunizing power The first deliberate attempt to attenuate a virus was Pasteur's work with the rabies virus But the value of the results with this virus has never been as well established as that of a naturally attenuated virus which had been previously discovered by the English physician, Jenner who made the observation that milkmaids who in the course of their work had contracted cowpox, which acts like an attenuated form of smallpox, were immune to smallpox Vaccination with this attenuated virus produces no serious disease, but does give immunity to smallpox Such vaccination, if repeated every five or ten years, will keep people immune to smallpox Where the measure has been thoroughly and energetically used, it has in fact practically stamped out the disease It is rather ironical that the most successful vaccine we have for a virus disease was the first one discovered

ANIMAL PARASITES

Animal parasites, or their eggs, may be ingested directly with food or may be transmitted by an intermediate host (the mosquito, for example, transmits the malarial parasite) In still other cases intricate life cycles have

been evolved, as in *Schistosoma mansoni*, whereby a free swimming form of the parasite is produced from the intermediate host and finds its way into water in which the human victim is exposed to it There it reaches the host, penetrates the skin by the action of hyaluronidase, and by active motion eventually reaches whatever part of the body it finds most suitable for its development

Animal parasites contain antigenic proteins and polysaccharides Some parasites possess anti-enzymes or other protective devices to keep them from being destroyed by the enzymes of the host As a general rule an attack by animal parasites does not confer immunity as effective as that conferred by bacterial or viral infections Superinfection or reinfection is often possible, as for example in malaria

Immunity to Animal Parasites

Circulating antibodies have often been detected in cases of parasitic infestation, although it has been more difficult to demonstrate them in the case of parasites living in the alimentary canal—possibly because less intact antigen from these parasites ever reaches the circulation to stimulate the production of antibodies

During World War II attempts were made to work out a method of vaccinating against malaria, and some success was achieved in monkeys and birds but the mixtures used were too toxic to be used in human beings Consequently, at the present time our main defense against malaria is the use of chemotherapeutic agents

In the case of some parasites such as *Trichinella spiralis* it has been definitely demonstrated that infected animals have a considerable degree of resistance to reinfection In other diseases such as schistosomiasis, the resistance to repeated infections seems to be very slight, and there is evidence that persons completely cured of the infection can be readily reinfected

METABOLITE ANTAGONISM

When late in the 19th century it became known that many diseases were the result of microbial invasion, attempts were made to find substances which would kill or restrain the invading organisms without doing serious damage to the host With few exceptions, this search was fruitless for four decades One notable exception was quinine, which was already known and empirically used in the treatment of malaria Another was the use of mercury, bismuth compounds, and organic arsenicals in syphilis and other spirochetal diseases The use of merc[illegible] dated the knowledge of the microbial nature of [illegible] by abc[illegible] r s, but the organic arsenicals were [illegible] as a result of serious and

extensive research by Paul Ehrlich with the defined objective of finding a drug which would rid the body of spirochetes

The *sulfonamides* became established during the 1930's as effective antibacterial agents against streptococcal gonococcal, meningococcal, and several other types of bacterial infection. The first of these drugs to be introduced was an organic dyestuff. Tréfouel and his collaborators soon demonstrated that the active group in this substance was sulfanilamide (formula II).

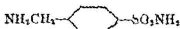
Clinical use of sulfanilamide and of its substitution products on the amide nitrogen led to the observation that their antibacterial action was inhibited by cell or tissue extracts or hydrolysates as for example by pus. Woods (20) studied yeast hydrolysates which inhibited the action of sulfanilamide and obtained strong presumptive evidence that the inhibitor was *p aminobenzoic acid* (formula III). One mol of *p* aminobenzoic acid was found to inhibit the antibacterial effect of 5 000 or more mols of sulfanilamide. Woods proposed as a working hypothesis to explain the action



II Sulfanilamide



III *p*-Aminobenzoic acid



IV Homosulfanilamide

of the sulfonamide drugs that (a) *p* aminobenzoic acid or a closely related substance is essential for the growth of sulfonamide sensitive bacteria, and (b) sulfonamides by reason of their structural similarity to *p* aminobenzoic acid compete for the enzyme system which normally utilizes *p* aminobenzoic acid in the bacterial cells.

This working hypothesis has not turned out to be a full explanation of all the antibacterial activities of the sulfonamide drugs, as is sharply indicated by the fact that *homosulfanilamide* (formula IV) is an effective antibacterial drug but is not inhibited by *p* aminobenzoic acid. Woods's work however, has stimulated many investigations into the general subject of metabolite antagonism the prevention of the normal utilization of a substance by the presence of an antagonist, which is usually a substance showing close similarity of structure.

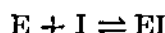
Lampen and Jones related the need for *p* aminobenzoic acid to its utilization in the *formation of folic acid* (14). Sulfonamides competitively prevent the incorporation of *p*-aminobenzoic acid into the folic acid molecule. Most organisms, possibly all organisms, require folic acid. Some require it ready made; these organisms are not sensitive to sulfonamides, since they do not build folic acid. The growth of such organisms can be

antagonized by certain structural analogues of folic acid, such as aminopterin The organisms which are characteristically sensitive to the sulfonamides are those which utilize p aminobenzoic acid in the synthesis of the requisite folic acid The inhibition of growth produced in such organisms by sulfonamides can be released either *competitively* by p aminobenzoic acid, or *non competitively* by folic acid

In any competitive inhibition—



$$E + S \rightleftharpoons ES \rightarrow E + P$$



$$E + I \rightleftharpoons EI$$

where E is the enzyme involved, S is its normal substrate, I is the competitive inhibitor, usually a structural analogue, and P is the product By the mass action law the dissociation constant of the enzyme substrate complex is K_S

$$K_S = \frac{[E][S]}{[ES]}$$

and the dissociation constant of the enzyme inhibitor complex is K_I

$$K_I = \frac{[E][I]}{[EI]}$$

Dividing K_I by K_S we can get an inhibition constant, K

$$K = \frac{[I][ES]}{[S][EI]}$$

At 50 per cent inhibition

$$[ES] = [EI]$$

and

$$K = \frac{[I]}{[S]}$$

Thus the effectiveness of a competitive inhibitor depends upon the *ratio* of concentrations of inhibitor and normal substrate, and not upon their absolute amounts

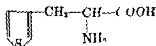
If enough folic acid is used to supply the full growth requirement, there will be no inhibition whatever of *Streptococcus faecalis* by sulfonamide There are however, other organisms which are sensitive to sulfonamides but their sen

described by

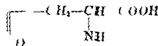
Most sulfona

While keeping within the bounds of his original hypothesis, Woods (21)

has offered several possible explanations for the failure of folic acid to release all sulfonamide sensitive organisms from sulfonamide inhibition (a) some organisms may not be able to assimilate preformed folic acid, (b) the final substance normally synthesized may be different from the synthetic folic acid used experimentally—for example, certain organisms might produce the citrovorum factor (see page 697) without folic acid being even an intermediate, (c) p-aminobenzoic acid may have essential functions in some organisms not involving the synthesis of folic acid This last point is borne out by the fact that certain nucleic acid derivatives, e g , thymine, adenine, guanine, and xanthine, and a number of amino acids including methionine may with some organisms replace p amino benzoic acid either as an essential metabolite or in releasing the organism from sulfonamide inhibition It seems reasonable to consider these substances as products of the utilization of p-aminobenzoic acid by the bacterial cells, and that their formation is inhibited by sulfonamides In cultures of *Escherichia coli*, maintained under sulfonamide inhibition an amine accumulates which could be converted to adenine by the addition of a one



V Thienylalanine



VI Furylalanine

carbon fragment followed by ring closure and amination in position 6 Evidence exists, and is summarized briefly by Woods (21) indicating that substances related to folic acid are concerned in the synthesis of a limited number of amino acids and in the formation of peptide linkages

Competitive inhibition of the growth of micro organisms need not involve components of enzyme systems Competitive antagonists to intermediates of carbohydrate metabolism and to amino acids, have been prepared and demonstrated For example, two effective inhibitors of the growth of *Escherichia coli* are thienylalanine (formula V) and furylalanine (formula VI) These substances are obviously related structurally to phenylalanine, and inhibition of bacterial growth by either of these substances is released by phenylalanine A constant ratio of inhibitor to phenylalanine is required to obtain 50 per cent inhibition, indicating that the inhibition is competitive (9)

Similarly, several structural analogues have been synthesized which are inhibitory for individual members of the vitamin B complex (formula VII)

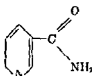
ANTIBIOTICS

The use of chemical substances to kill or inhibit pathogenic micro organisms within the body of the host is chemotherapy The chemotherapeutic agents so far named have been products of chemical synthesis, with the

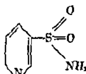
exception of quinine the laboratory synthesis of which was not announced until 1951 Those chemotherapeutically effective substances which are products of *living organisms (usually, but not always micro organisms)* have been designated as *antibiotics* Many of these have been shown to operate by metabolite antagonism but some by altogether different mechanisms

It was noted by early workers in bacteriology that certain organisms

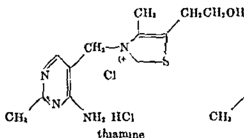
Food Factors | *Inhibitory Analogues*



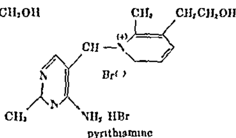
nicotinamide



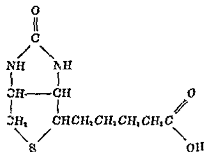
pyridine-3-sulfonamide



thiamine

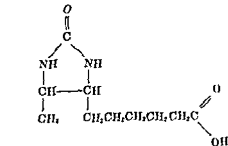


pyrithiamine



biotin

VII



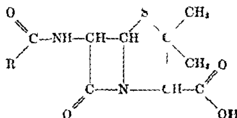
desthiobiotin

such as the pyocyaneus bacillus (*Ps aeruginosa*), would successfully invade and overgrow certain other microbial species *in vitro* It could even be demonstrated that pyocyaneus would overgrow anthrax in the living body of an experimental animal Such pitting of one living organism against another did not develop into a useful form of therapy, nor did the pyocyaneus bacillus ever become a source of useful antibiotics Such antibacterial substances as pyocyaneus produces and they are numerous are too toxic to the animal body for effective use

The earliest recorded use of antibiotics had nothing to do with the treat

ment of human disease, and took place without benefit of bacteriological science Early in the 16th century, English brewers learned from their colleagues in the Low Countries that *hops* when added to wort, resulted not only in a better flavor but also in a more dependable fermentation and better keeping qualities "The continuance of the drink is always determined after the quantitie of hops, so that being well hopped it lasteth longer," wrote Harrison in his late 16th century *Description of England* The resin acids of hops are quite specifically inhibitory to the growth of several species of gram positive bacteria which, in the absence of hops, tend to multiply in competition with yeast in the fermentation stage of brewing (2) The mechanism of the inhibition has not been explained

Penicillin was the first truly successful medical antibiotic and is still widely used It was discovered in 1929 by Fleming, who observed the death of staphylococcal colonies near a contaminating colony of *Penicillium*



VIII Penicillin—general formula

notatum, and who also observed the antibacterial action of culture filtrates of the same mold

There are actually several penicillins organic acids for which we may write a general formula (formula VIII) in which R stands for a benzyl group in the penicillin most commonly used (Penicillin G) Other penicillins obtainable from cultures of *P notatum*, or of *P chrysogenum*, may have in place of the benzyl group *p* hydroxybenzyl, Δ^2 pentenyl, *n* amyl or *n* heptyl

Penicillins inhibit, and in higher concentrations kill, growing cells of susceptible strains Action on non growing or resting cells is insignificant Cells of *S aureus* take up penicillin and bind it firmly Free nucleotide begins to increase in growing cells immediately after the binding of penicillin In tests made using radioactive penicillin it was found not to be removable from the cells either by washing or by equilibration with non radioactive penicillin (16a) Penicillin does not penetrate into yeast cells, which are not affected by its presence

Gram positive bacteria as a group are more susceptible to penicillins than gram negative bacteria Staphylococci, which are gram positive, can

Neomycin is a water soluble, thermostable, basic substance obtained from filtrates of *Streptomyces fradiae* The main interest in the substance is its activity against streptomycin resistant microbes, including the resistant strains of *M tuberculosis*

Numerous other antibiotics have been characterized and introduced into clinical practice The chemical structure of some of these (e g , chloramphenicol (formula X)) has been determined and chemical synthesis accomplished Others (e g bacitracin) have been found to be polypeptides, which have so far remained unsynthesized except by microbial action

REFERENCES

1 ABRAMSON H A *et al* The specificity of the second stage of bacterial agglutination J Bact 50 15–22 1945
2 BISHOP I R The resins of hops as antibiotics Symp Soc Exptl Biol III *Selective Toxicity and Antibiotics* pp 101 104 New York N Y, Academic Press
3 BJØRNEBOE M The specific protein in rabbit antipneumococcal serum and its relation to the increase of serum protein during immunization J Immunol 37 201 206 1939
4 BORDET J *Traite de l Immunité* Paris Masson & Cie 19?0
5 BOYD W C AND BERNARD H Quantitative changes in antibodies and globulin fractions of rabbits injected with several antigens J Immunol 33 111 122 193?
6 BOYD W C *et al* The heat of an antibody-antigen reaction J Biol Chem 139 787 791 1941
7 BOYD W C AND PURNELL M A The essential difference between the two optimum proportions ratios J Exper Med 80 289–298 1944
8 BURNET F M *Virus as Organism* Cambridge Mass Harvard University Press 1945
9
10
1937
11 GALE L F AND RODWELL A M Amino acid metabolism of penicillin resistant staphylococci J Bact 55 161 167 1948
12 HEIDELBERGER M Quantitative absolute methods in study of antigen antibody reactions Bact R ?5 1939
13 KAHN R L *The Kahn Test*, The W ... *Co* 1928
14 LAMPEN J O AND JONES ... *tagonism* inhibition of certain lactobacilli and e ... pteroylgl ... related compounds J Biol Chem
15 LEVI ... phil s ... rence to the Mem ... s Ge ... Coun
16 MARRA ... chem ... cil ... Bact
16a MA ... NSON
57

17 NEURATH H The apparent shape of prote molecules J Am Chem Soc 61 1841 1844 1939

18 PAULING L A theory of the structure and process of form t n of antibod es J Am Chem Soc 62 2643 265 1940

19 UMBREIT W W et al The act on of strep ton c n X The fo n at on of c rate J Bact 61 595-604 1951

20 WOODS D D The relat on of p-am nobenzo c ac d to the mechan sm of the ac t on of sulphanilam de B t J Exper Pa h 21 74-90 1940

21 WOODS D D B ochem cal s gn ficance of the ompet t on be we p am nol en zoic ac d a d the s lf amides Ann New York Acad Sc 52: 1199-1211 1950

PART VI

Appendices



APPENDIX I

Colloids and Colloid Phenomena

In 1861 Thomas Graham proposed the distinction between crystalloids and colloids, the crystalloids being characterized by a tendency to form crystals when separating from a water solution and the colloids by a tendency to separate out in the form of gelatinous or amorphous masses (the word colloid being derived from the Greek word for glue). Graham found the crystalloids to diffuse readily through membranes such as parchment or a pig's bladder while the colloids diffused slowly or not at all. Graham thought this difference in diffusibility plus a tendency of the colloids to form aggregates consisting of several or many molecules were the two chief differences between crystalloids and colloids.

Later workers studied the physical and chemical behavior of colloids and a whole science known as "colloid chemistry" developed. Empirical equations for the reaction between colloids and simple chemicals were proposed and it was asserted that since only the surface of the colloid could react the ordinary laws of stoichiometric chemistry could not be expected to apply to these reactions.

This state of affairs has gradually been modified by two rather opposing trends. One is the trend largely initiated by Jacques Loeb (10) and carried on by researchers such as Svedberg (21) to regard all colloidal phenomena as explainable by the fact that the molecules of colloids are big; certainly their nondiffusibility is explained by this fact. Some modern writers, especially certain physical chemists, are rather skeptical about the existence of any such science as colloid chemistry and any such substances as colloids. Another trend is exemplified by numerous writers who have done so much work with colloidal phenomena that they would no more doubt the existence of colloidal phenomena than they would doubt their own existence (1, 6, 8, 11). Which of these schools of thought is right?

The answer, as is so often the case when scientific controversies are examined in the light of later knowledge, is that both schools are partly right

5 *Electric charges on micelles* If the constituent atoms or molecules of a micelle are considered to be firmly bound together, but the whole micelle permeable to the smaller inorganic ions, there should be a Gibbs Donnan effect even in the absence of any real membrane, for the colloidal components of the micelle, being held together firmly, are just as effectively prevented from migrating as if they were surrounded by a semipermeable membrane Loeb showed how the Gibbs Donnan equilibrium would account for the charges observed on protein particles in suspension There is little doubt it applies to many other sorts of micelles also

6 *Osmotic pressure* If the colloidal molecule or micelle can not diffuse through a membrane with which it and other diffusible ions are in contact, there will be a difference in osmotic pressure, which has already been discussed (see page 32) The magnitude of this pressure will depend partly on the pH of the solution, and partly on the other ions present Loeb found good agreement between the values observed and those calculated from the Gibbs Donnan equilibrium

7 *Surface phenomena* Whether or not all the atoms of a colloid micelle can be reached by a smaller atom or ion, and whether or not a reaction between the two can take place within the micelle as well as on the surface depends on the size of the ion, its charge, and the porosity of the micelle In some cases measurement shows that penetration into the micelle is impossible or incomplete, in other cases, for example the decomposition of ammonia by certain catalysts, the inside of the particles of catalyst is able to participate about as effectively in the reaction with ammonia as does the outer surface

In other cases however, this is not true, and some substance in the solution tends to be concentrated at the surface of a micelle There seem to be a number of examples of this in the chemistry of living organisms As happened so often, Gibbs (7) treated the situation mathematically before adequate experimental data were at hand His equation can be written

$$U = -c/RT(d\delta/dc)$$

where U is the excess of substance in the surface layer of the micelle, c is the concentration of the substance in the bulk of the liquid, δ the surface tension, R is the gas constant, and T the absolute temperature

The equation of Gibbs has been confirmed experimentally Donnan suggested a method involving the measurement of volumes of bubbles of gas through a solution containing some substance which is adsorbed at the surface of the bubbles Fair agreement between experiment and prediction was obtained McBain suggested another method in which the surface layer of a long trough was actually shaved off and analyzed A great number of determinations carried out by this method gave excellent agreement with the predicted results

The prominence of adsorption effects at surfaces is often so marked that some have called colloidal chemistry "surface chemistry." Various materials are observed to be concentrated at the surface of adsorbants such as charcoal, various metallic oxides, and silica gel.

Various equations have been proposed to explain the quantitative course of adsorption. The best known is probably the empirical equation of Freundlich (6) which can be written

$$R = kx^n$$

where R is the amount of the substance absorbed per unit of adsorbant, x is the final concentration of the adsorbed substance in the supernatant, and k and n are constants.

Another well known adsorption equation is that of Langmuir which was derived from kinetic considerations. It may be written

$$R = nkx/(1 + kx)$$

where R is the amount of adsorbed substance combining with a fixed amount of adsorbant, n and k are constants, and x is the final concentration of the adsorbed substance.

This equation fits many adsorption data, but Hitchcock (9) has shown that a simple transformation of the law of mass action gives an equation of the same form, so it can not be concluded from the fact that a reaction follows the form of this equation that it is an "adsorption" reaction and not a strictly stoichiometric one.

An examination of immunochemical data (2) which involve reactions now generally believed to be purely chemical in nature, although probably confined to the surface of the molecules, shows that the data conform, sometimes better to the Freundlich equation, sometimes to that of Langmuir. There can be no doubt that either equation may be applicable to at least some experiments or to part of the range of a single experiment.

Optical methods in colloid chemistry. The micelles of colloids are too small to be seen by the ordinary microscope. They are nevertheless larger than the common molecules and ions which Graham called crystalloids, and Zsigmondy devised the first method of rendering them visible. Optical theory had shown that the best microscopes could never resolve any object whose smallest dimension was less than about one half the wave length of the light used.[1] The human retina, being sensitive to radiations only

[1] Strictly the relation is

$$h = \frac{0.61T}{N.A.}$$

where h is the smallest distance by which two particles can be separated and still be resolved as two particles, T is the wave length of the radiation, and N.A. is the numerical aperture of the microscope.

down to about 400 millimicrons, is the limiting factor in direct visual microscopy Photographs could be taken by ultraviolet light, using micro scopes made with quartz lenses, but this only increased the resolving power by something like a factor of two, and still did not show up the micelles of colloids However, if a beam of visible light is passed through a solution which contains suspended particles having dimensions of the same order of magnitude as the wave length of light, each little particle in the sus pension scatters the light which strikes it Each particle, in fact, acts as a secondary source of light, and sends some off in all directions Siedentopf and Zsigmondy, therefore arranged a microscope (17) so a beam of visible light was passed through the solution horizontally None of this light reached the microscope directly But observation from above enabled the scattered light which happened to be coming directly upwards to be seen, and in colloidal suspensions and solutions minute points of light were seen, whereas true solutions such as those of NaCl or alcohol showed a perfectly dark field to the observer This new arrangement was called the ultramicroscope, and was the first visual demonstration of the micelles which exist in colloidal solutions

More recent workers have taken advantage of the fact that much can be inferred as to the size and shape of the submicroscopic particles by measuring the amount of light scattered at various angles with respect to the incident beam and light scattering measurements are becoming one of the important tools in the study of molecules and aggregates of about the dimensions of proteins (4 14)

In the meantime, the development of the quantum theory had led to the view that radiation could be considered either as waves or as particles In fact neither view alone will explain all the phenomena which are observed with radiation It was predicted, and verified experimentally, that electrons at first considered as particles, ought also to behave as waves under suitable conditions The wave length corresponding to an electron is much less than even the shortest ultraviolet (about 2 to 3 millimicrons as opposed to 75 with the best ultraviolet microscopes) This has made it possible to photograph submicroscopic particles such as viruses and even some of the larger protein molecules (16, 19) The electron microscope thus has about the right resolving power to enable colloidal molecules and *micelles to be photographed* (1, 22)

REFERENCES

1 ANDERSON T F The study of colloids with the electron microscope Advances Colloid Sc 1 353-390 1942
2 BOYD W C *Fundamentals of Immunology* 2nd edit New York N Y Interscience Publishers 1947
3 BOYD W C AND BEHNKE J Aggregation in solution of a synthetic hapten Science 100 13 14 1944

4 DOTY P AND EDSALL J T Light scattering in protein solutions Advances Protein Chem 5 35–171 1951
5 FEVOLD, H L Egg proteins Advances Protein Chem, 6 18 ..2 1951
6 FREUNDLICH H *Kapillarchemie* Leipzig Germany, Akad Verlags Ges (2 edit) 1927
7 GIBBS, J W *Collected Works* New York N Y Longmans Green & Co 1928
8 GUSTAVSON K H Some protein-chemical aspects of tanning processes Advances Protein Chem 5 354–421 1949
9 HITCHCOCK D I Notes The formal identity of Langmuir's adsorption equation with the law of mass action J Am Chem Soc 48 2870 1926
10 LOEB J *Proteins and the Theory of Colloidal Behavior* New York N Y McGraw Hill Co 1922
11 LUNDGREN H P Synthetic fibers made from proteins Advances Protein Chem 5 305–351 1949
12 NORTHROP J H Crystalline pepsin isolation and tests of purity J Gen Physiol, 13 739–766 1930 Crystalline pepsin general properties and experimental methods *Ibid*, 767 780
13 NORTHROP J H *et al* *Crystalline Enzymes* New York, N Y Columbia University Press, 1948
14 OSTER G Two phase formation in solutions of tobacco mosaic virus and the problem of long range forces J Gen Physiol 33 445–473 1950
15 OSTWALD C W W *in* KUHN A K *Kolloidchemisches Taschenbuch II* Leipzig Akad Verlags 1935
16 POLSON A AND WYCKOFF R W G Shape of haemocyanin molecules Nature 160 153 1947
17 SIEDENTOPF H AND ZSIGMONDY R Ann Physik (4) 10: 1 39 1903
18 STANLEY W M Chemical studies on the virus of tobacco mosaic VII An improved method for the preparation of crystalline tobacco mosaic virus protein J Biol Chem 113 673–678 1936
19 STANLEY W M AND ANDERSON T F Electron micrographs of protein molecules J Biol Chem 146: 25–30 1942
20 SUMNER J B AND SOMERS G F *Chemistry and Methods of Enzymes* 2nd edit New York N Y Academic Press 1947
21 SVEDBERG T AND PEDERSEN K O *The Ultracentrifuge* London England Oxford University Press 1940
22 WYCKOFF R W G The electron microscope of macromolecules Advances Protein Chem 6 1–33 1951

APPENDIX II

Isotopes

Perhaps no technique of investigation has been so unexpected at the time of its first development, so powerful a tool after development, and so revolutionary in its immediate applications as that of isotopic tracer research in biochemistry. Its particular efflorescence in these initial years of the atomic age would make any textbook on human metabolism incomplete that did not contain fairly extensive reference to this unparalleled device for probing the metabolic pathways of the human body. The understanding of tracer methodology must begin with a consideration of the atomic nucleus.

THE ATOMIC NUCLEUS

Nuclear Structure

The atom is composed of two parts: a compact central *nucleus* carrying unit positive charges of varying number, and a number of "planetary" *electrons*—each carrying a unit negative charge. In the neutral atom the number of electrons is equivalent to the number of positive charges in the nucleus so that the atom as a whole is uncharged. Ordinary chemical reactions involve the outermost electron shells of an atom (see Chapter 5), and the chemical properties of individual elements depend upon the number and configuration of these outer shells. Ions are atoms which have lost or gained one or more electrons, acquiring positive or negative charges, respectively.

The nucleus is a very small portion of the atom in terms of volume; the diameter of an atom is of the order of 10^{-8} centimeters, whereas the nucleus is about 10^{-13} cm in diameter. The volume of the nucleus is thus only 10^{-15} that of the entire atom. Nevertheless, almost all the mass of the atom is concentrated in that tiny nucleus, since the electrons—the wave packets of which fill virtually all the atom—possess very little mass indeed. It is this fact that lies behind the frequently heard statement that an atom is mostly empty space. The simplest atom is hydrogen, one form of which possesses the lightest known nucleus and only a single planetary electron. It has 99.94 per cent of its mass in the nucleus. The most complex atom known is californium, whose nucleus is more than 240 times as massive

as that of hydrogen and which has 98 electrons, having 99 98 per cent of its mass in the nucleus

The atomic nucleus is with one exception, a composite structure, it is composed of still smaller entities of two kinds *protons* and *neutrons* In terms of mass these are about equal The proton, however has a unit positive charge—that is a charge capable of neutralizing exactly that of one electron, while the neutron is uncharged With the exception of the simplest known nucleus that of ordinary hydrogen which consists of a single proton and is the only known non composite nucleus all atomic nuclei contain both protons and neutrons

For a given element the number of protons in the nucleus is fixed and is equal to the *atomic number* of that element The atomic number of an element is defined as the number of unit positive charges in the nuclei of its atoms, which depends of course upon the number of protons within the nucleus The atomic number of an element may also be defined as the number of planetary electrons present in the neutral atom

For any given element the number of neutrons in the nucleus may vary within narrow limits A variation in the number of the neutrons does not affect the chemical properties of the element since neutrons are uncharged and therefore do not by addition to or subtraction from the nucleus alter the positive charge on the nucleus

The atomic weight of a particular nucleus (or *nuclide*) depends upon the sum of the number of protons and neutrons (together called *nucleons*) The mass of all atomic and subatomic particles is based upon the arbitrary assignment to oxygen of a mass number of exactly 16 a value which is treated as a pure number On this basis the neutron and the proton have mass numbers of very nearly 1 More exactly the proton has a mass number of 1 00813, while that of the neutron is 1 00896 and other atomic particles likewise differ by a fraction of a per cent from integral mass numbers These small differences while rendering possible the atomic bomb are of no importance in biochemistry and mass numbers will be for the large part treated as though they were exact whole numbers The electron has a mass number of about 0 00054, so that in most cases no serious error is introduced if its mass number is considered as simply zero

The mass number of a nucleus is usually symbolized as A, while the atomic number is referred to as Z A little thought will show that the number of protons in a given nucleus is equal to Z while that of the neutrons, to $A - Z$

Isotopes

It has already been said that the neutrons in the nucleus of a given atom may vary in number Although this variation does not affect the chemical nature of the atoms in any general way it does introduce important dif-

ferences in their physical properties Isotopes are atomic species the nuclei of which are composed of a fixed number of both protons and neutrons, while in an element, as has been said, only the number of protons within the nuclei are fixed Most elements are found to exist in nature as mixtures of different isotopes

This may perhaps be understood more clearly if a specific case is considered—that of hydrogen Most neutral atoms of hydrogen consist of a single proton as the nucleus and a single electron balancing its charge The mass number of such a hydrogen atom is 1 if we consider that of the proton to be 1 and that of the electron to be 0 In any sample of hydrogen which may be obtained by ordinary chemical means from any source 99 98 per cent of the atoms have the configuration just described (Certain procedures are known to the physical chemist which alter this percentage, but none of these comes under the classification of ordinary chemical means) The remaining 0 02 per cent of the hydrogen atoms possesses nuclei consisting of two particles one proton and one neutron Since the nuclear charge is still 1, such an atom would still have but one electron in its neutral state and would still be, chemically hydrogen Its mass number, however, would be 2, double that of the more common variety of hydrogen A sample of hydrogen consisting only of atoms of the heavier variety would be twice as dense under similar conditions of temperature and pressure as would ordinary hydrogen Such heavy hydrogen" has been prepared and its physical properties such as melting point, boiling point specific heat, and so on, have been found to be quite different from those of ordinary hydrogen Thus, the boiling points in degrees absolute for these hydrogen isotopes are 20 38 for the lighter one and 23 6 for the heavier

The difference in properties between these isotopes is unusually great considering that they are isotopes of a single element This is because isotopes never vary in mass number by less than 1 In the case of hydrogen the mass number is only 1 to start with and even the smallest possible difference in isotope mass number represents a *100* per cent *increase in* mass In the case of oxygen, most of whose atoms have a mass number of 16, an isotope with a mass number of 17 would be less than 7 per cent more massive As atoms become still more complex the percentage differences between isotopes become continually smaller

Such are the differences between the two isotopes of hydrogen that they are usually given different names and symbols The lighter isotope is known by the common name *hydrogen*, or more rarely *protium* while the heavier isotope is called *deuterium*, and is often symbolized as D There is still a third isotope of hydrogen very rare and including only one atom in ten million or so, which has a mass number of 3, since its nucleus is composed of one proton and two neutrons This 'super heavy" hydrogen

TABLE 46

Naturally occurring isotopes of elements of biochemical interest

ELEMENT	NUMBER PROTONS (Z)	NUMBER NEUTRONS (A − Z)	MASS NO (A)	PER CENT OCCURRENCE WITHIN ELEMENT
Hydrogen	1	0	1	99 98
Hydrogen (deuterium)	1	1	2	0 02
Hydrogen (tritium)	1	2	3	trace
Carbon	6	6	12	98 9
Carbon	6	7	13	1 1
Nitrogen	7	7	14	99 62
Nitrogen	7	8	15	0 38
Oxygen	8	8	16	99 76
Oxygen	8	9	17	0 04
Oxygen	8	10	18	0 20
Fluorine	9	10	19	100 00
Sodium	11	12	23	100 00
Magnesium	12	12	24	78 6
Magnesium	12	13	25	10 1
Magnesium	12	14	26	11 2
Phosphorus	15	16	31	100 00
Sulfur	16	16	32	95 06
Sulfur	16	17	33	0 74
Sulfur	16	18	34	4 18
Chlorine	17	18	35	75 4
Chlorine	17	20	37	24 6
Potassium	19	20	39	93 3
Potassium	19	21	40	0 01
Potassium	19	22	41	6 7
Calcium	20	20	40	96 96
Calcium	20	22	42	0 64
Calcium	20	23	43	0 15
Calcium	20	24	44	2 06
Iron	26	28	54	5 84
Iron	26	30	56	91 61
Iron	26	31	57	2 17
Iron	26	32	58	0 31
Cobalt	27	32	59	100 00
Copper	29	34	63	69 1
Copper	29	36	65	30 9
Zinc	30	34	64	48 89
Zinc	30	36	66	27 81
Zinc	30	37	67	4 11
Zinc	30	38	68	18 56
Zinc	30	40	70	0 62
Iodine	53	74	127	100 00

is called *tritium*, and is sometimes symbolized as T The isotopes of no other chemical element are assigned separate names or symbols

A list of naturally occurring isotopes of elements of biochemical interest with their nucleon contents and frequency of occurrence is contained in table 46, the data of which were obtained from Sullivan s chart (2)

Isotopic differences persist when atoms form part of molecules Water, for instance, may contain protium, deuterium, or tritium atoms so that the following compounds may be formed H_2O HDO, HTO, D_2O, DTO, T_2O Since three stable isotopes of oxygen exist, with mass numbers of 16, 17, and 18, it can be seen that *three series of such kinds of water exist* making a total of 18 in all The water most commonly found in nature is that consisting of two protiums and an oxygen 16, with a molecular mass number of 18 (The molecular weight of a compound would more properly refer to the average molecular mass numbers of the various isotopic combinations existing within a compound In substances of biochemical interest the molecular weight is very close to the molecular mass number of the most common isotopic combination) Although the most massive water molecule that exists is one containing two tritiums and an oxygen 18, with a molecular mass number of 24, it is D_2O (sometimes called deuterium oxide), with a mass number of 20, which is commonly referred to as *heavy water* The difference in mass numbers between ordinary water and heavy water is a little over 11 per cent, which is great enough to result in very easily measured changes in the physical properties of water Thus, the boiling point, melting point and maximum specific gravity of ordinary water are 100°C, 0°C, and 1 0000 while the corresponding figures for heavy water are 101 4°C, 3 82°C, and 1 1071

A systematic symbolism for isotopes involves the use of a subscript before the symbol of the element to indicate the atomic number and a superscript after the symbol to indicate the mass number A generalized such symbol would $_Z X^A$ As specific examples we might put forward the case of protium deuterium and tritium which in this notation would be $_1H^1$, $_1H^2$, and $_1H^3$ Similarly the two carbon isotopes would be $_6C^{12}$ and $_6C^{13}$, while the three oxygen isotopes would be $_8O^{16}$, $_8O^{17}$, and $_8O^{18}$ Since each element has an invariant atomic number, the initial subscript is not necessary and there is no ambiguity in referring simply to H^2 or C^{13} Or, for that matter, the chemical symbol may be left out and only the two numbers given In the biochemical literature the first of the two simplified notations is most frequently found

Nuclear Stability

The atomic nucleus has within it the potentialities of instability It contains only one kind of charged particle, the proton, and by the ordinary

laws of electrostatic attraction one would expect that these protons would repel one another and that a structure composed only of positive charges would not exist Actually, no atomic nucleus consists of more than one proton without the addition of neutrons as well. Although neutrons are uncharged, the laws of electrostatic attraction and repulsion between charges at distances comparable to those within an atomic nucleus (which are 10^6 times less than those which occur anywhere else in nature) are quite different from those met with elsewhere Apparently at such short distances, various arrangements of protons and neutrons can become stable aggregates The nature of the attractive forces between nucleons is a major field of investigation among nuclear physicists today and is beyond the scope of this book We need only know that for a given number of protons in a nucleus only a certain number of neutrons will when present, yield a stable aggregate

In the case of certain nuclei, usually among those containing an odd number of protons, the number of neutrons required for stability is fixed and can not vary Examples of such elements are sodium whose 11 protons must be joined with 12 neutrons no more and no less for stability, phosphorus, whose 15 protons must be joined with 16 neutrons, and iodine, whose 53 protons must be joined with 74 neutrons Other nuclei, particularly those with an even number of protons are more liberal in their neutronic requirements Tin with 50 protons possesses stable nuclei of ten different mass numbers containing 62, 64, 65, 66, 67, 68 69, 70 and 72 neutrons, respectively This is another way of saying that tin has ten stable isotopes

Not all stable proton neutron combinations are equally stable, if we judge by the frequency with which such combinations occur in nature Thus, as we see from table 46 99 76 per cent of all oxygen molecules contain nuclei with eight protons and eight neutrons While those nuclei containing eight protons and nine or ten neutrons are also stable in that they will exist unchanged indefinitely unless subjected to subatomic bombardment, they must have been less readily formed—i e, presented less economical configurations from an energic viewpoint in the early formative times of the universe The majority of the elements, even when they possess a number of isotopes are predominantly in the form of one particular, and presumably, most stable isotope This is particularly true among the simpler elements with chlorine as an exception since two isotopes of that element exist in the ratio 3 1, and boron whose two isotopes are distributed in the ratio of 4 1

The ratio of distribution of the various isotopes within an element is, with very minor variations, constant in all known samples of that element. Thus the O^{17} content of oxygen is the same for atmospheric oxygen and

for the oxygen contained in the water molecules of the ocean and in the silicate molecules of the soil This constancy is not even confined to earth, since elements found in the meteorites show the same isotopic ratios as the corresponding elements on our planet This constancy of isotopic constitution is an important factor in tracer work

Among the first twenty elements of the atomic table the most stable proton neutron combinations are frequently those where the number of each is equal Thus the most common isotope of helium has two protons and two neutrons, that of carbon six of each, that of nitrogen seven of each, of oxygen eight of each, of magnesium twelve of each, of sulfur sixteen of each, and of calcium twenty of each Exceptions exist, particularly among the nuclei containing odd numbers of protons, where the number of neutrons is one greater (as in sodium with 11 protons and 12 neutrons, or phosphorus with 15 and 16), or in the case of hydrogen, one less

In general the most stable nuclei tend to be those containing an even number of protons and an even number of neutrons Examples of these are He^4, C^{12}, O^{16}, Mg^{24} Si^{28}, and S^{32} Conversely, those nuclei with odd numbers of both protons and neutrons are least stable, and among elements of biochemical interest the only important case of such a nucleus is that of N^{14} with seven of each Deuterium with one of each, and K^{40} with nineteen protons and twenty one neutrons are present in their elements only to one or two hundredths of a per cent

As the complexity of the nucleus increases and more and more protons are packed into its narrow limits the proportion of neutrons must be continually increased to maintain stability In table 46 we see that the most common isotope of iron has 26 protons and 30 neutrons (a neutron proton ratio of 1 15), while in iodine the only stable combination is 53 protons and 74 neutrons (a neutron proton ratio of 1 40) The most complex stable nucleus known is that of bismuth which contains 83 protons and 126 neutrons, a neutron proton ratio of 1 52 Apparently, when the number of protons in a nucleus is higher than 83 no quantity of neutrons will suffice for stability and stable nuclei more complex than bismuth do not exist

Radioactivity

Nuclei more complex than that of bismuth, although not stable do exist The neutron proton composition of those nuclei spontaneously changes, becoming eventually a stable form This change or decay" is the phenomenon called radioactivity Radioactive nuclei decay in one or more of three ways They may emit an alpha particle, consisting of two protons and two neutrons lowering the proton content and by decreasing their more numerous neutrons only an equal amount, increasing the neu

tron proton ratio The alpha particle is, actually, the nucleus of a helium atom and may be represented chemically as He^{++}, although it is more usually symbolized as the Greek α Once outside the confines of the radioactive nucleus the alpha particle will tend to capture two electrons from the surroundings and become gaseous helium For this reason, ores containing such radioactive elements as uranium and thorium frequently contain occluded helium as well

Another form of radioactive emission is the *beta particle* which is simply a high speed electron, and a third form of emission is the *gamma ray*, which is not a particle in the ordinary sense but an electromagnetic radiation just as ordinary light is, but much more energetic The wave length of gamma rays is much shorter than that of even the x ray and the radiation is correspondingly more energetic and penetrating The emission of *an electron from a nucleus containing only protons and neutrons represents* the change of a neutron into a proton the gain of a positive charge being equivalent to the loss or ejection of a negative charge in the form of an electron

A nucleus which emits an alpha particle loses four in mass number and two in atomic number One which emits a beta particle loses nothing in mass number but gains one in atomic number One which emits a gamma ray is unchanged in both respects By using a combination of such changes successively, a nucleus such as that of uranium can change to a somewhat simpler nucleus, which will itself change to another and so on, until a stable nucleus such as one of lead or bismuth is reached and the process ceases

These radioactive changes have two startling properties In the first place, a tremendous amount of energy is released per mass of material as the changes take place, far more energy than can be released by even the most energetic chemical reaction known to man Secondly, the rate at which the changes take place is unaffected by any change in temperature or pressure Two questions may therefore be raised, one for each of these properties First, where does the energy come from, and second, why are not all the radioactive nuclei long since decayed and gone?

Radioactive energy is derived from the supply within the atomic nucleus Just as molecules contain a store of chemical energy which was utilized in the formation of various electronic bonds between atoms (see page 142), so nuclei contain a store of *nuclear energy* in the more powerful bonds between protons and neutrons A rearrangement of nuclei into more stable configurations *involves of necessity the* release of some of this energy Nuclear energy is of an order so great that it can be detected as mass Einstein has shown that there is a definite mass-energy equivalence and that one gram of mass represents the equivalent in energy of 9×10^{20}

ergs The energy changes in ordinary chemical reactions represent changes in mass so small that our most delicate instruments can not detect them In nuclear changes, however, gains and losses of energy are large enough to upset the "law" of conservation of mass if not allowed for

The radioactive nuclei still exist simply because of the extremely low rate at which some of them change Such elements as uranium and thorium are almost but not quite stable U^{238} has a half life of nearly five billion years That means that of a given mass of uranium, half will decay in five billion years, half of that remaining will decay in another five billion years, and so on In the case of Th^{232}, the half life is even longer, nearly fourteen billion years Although the elements into which they decay are much more unstable with half lives that can be counted in centuries (radium is 1600 years), days (radon is four days), and split seconds (thorium C′ is one billionth of a second) uranium and thorium remain as unfailing sources of entire series of radioactive compounds Since the age of the universe has been estimated as a mere two to three billion years, it can be seen that more than half of the original uranium and more than four fifths of the original thorium exist A third series of radioactive compounds has as its long lived parent the now notorious U^{235}, whose half life—a mere nine hundred million years—is long enough to allow remnants of itself still to exist

What happens when no sufficiently long lived parent exists is indicated by the missing fourth series (there is room for four series all told, for reasons that we need not go into here) of naturally radioactive elements The most long lived member of that series is one of the isotopes of neptunium, Np^{237}, whose half life is a trifle of only two million years During the lifetime of the earth, neptunium has decayed away into an indetectable remnant and is now known only because man has learned to create nuclei artificially Among the daughter members of this series are what were referred to until World War II as the missing elements 85 and 87, which do not exist naturally but have since been synthesized and given the names of astatine and francium, respectively

Radioactivity is not confined to elements more complex than bismuth Any element, if the neutrons in its nucleus are increased or decreased below certain narrow limits, becomes radioactive Among the elements up to and including bismuth however, radioactive isotopes usually have comparatively small half lives so that any which may have existed have long since vanished A very few exceptions exist among naturally occurring nuclei, which although relatively simple in structure, have long half lives Among the elements of biochemical interest the best example is K^{40}, with a half life of about one and a half billion years It emits beta rays Tritium is also radioactive emitting weak beta rays It has a half life of only eleven years,

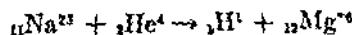
thus it would not be expected to exist naturally but it is, however, continually recreated in trace quantities in natural transmutative processes.

NUCLEAR REACTIONS

Transmutation

The radiations of radioactive materials are so energetic that if allowed to impinge upon other atoms they do not merely disturb the outer electrons and thus effect chemical changes as do such less energetic forms of radiation as visible light, but actually disturb the nucleus, initiating changes in its structure and sometimes disrupting it. Such nuclear changes amount frequently to changes in the nature of the element and represent the first true success in the old alchemists' dream of transmutation. Devices such as the cyclotron and the atomic pile are used to produce high concentrations of nucleons. In addition, deuterons (the nuclei of deuterium, consisting of one neutron-proton pair) are used.

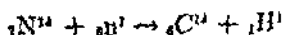
A typical nuclear reaction would be symbolized as follows:



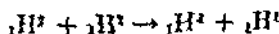
$$_{11}Na^{23} + {}_2He^4 \rightarrow {}_1H^1 + {}_{12}Mg^{26}$$

the significance of this is that an ordinary sodium atom when struck by alpha particles ($_2He^4$) absorbs the impinging particle and emits a proton ($_1H^1$), leaving behind a magnesium isotope ($_{12}Mg^{26}$). Note that in such a reaction the atomic numbers and the mass numbers as well add up to the same values on each side of the equation.

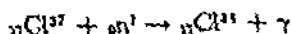
Other symbols used in writing nuclear reactions are $_1H^2$ for the deuteron, $_0n^1$ for the neutron. Electrons may be represented as $_{-1}e^0$ and positrons (very short lived "positive electrons" which are sometimes emitted by nuclei) as $_{+1}e^0$. A gamma ray is indicated by the Greek letter γ (just as electrons and positrons are sometimes symbolized by the Greek letter β, with a negative or positive superscript). The reader should be able to interpret without further explanation such nuclear reactions as



$$_7N^{14} + {}_0n^1 \rightarrow {}_6C^{14} + {}_1H^1$$

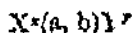


$$_1H^2 + {}_1H^2 \rightarrow {}_1H^3 + {}_1H^1$$



$$_{17}Cl^{37} + {}_0n^1 \rightarrow {}_{17}Cl^{38} + \gamma$$

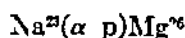
In general, the bombardment of atomic nuclei with these high-energy particles results in the emission by those nuclei of relatively simple particles and a simplified notation for such reactions has now arisen. The form of this notation may be described as



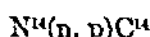
$$X^x(a, b)Y^y$$

which indicates that when the nucleus X with a mass number x is bombarded with a, it emits b, leaving behind the nucleus Y with the mass number y In this notation, the proton and deuteron are represented by the symbols p and d, while the alpha particle is represented as α Other symbols are the same as in the case of the more extended notation shown

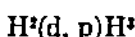
In the condensed notation the four nuclear reactions given would be represented as



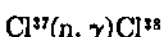
$$Na^{23}(\alpha\ p)Mg^{26}$$



$$N^{14}(n, p)C^{14}$$



$$H^{2}(d, p)H^{3}$$



$$Cl^{37}(n, \gamma)Cl^{38}$$

In at least one variety of nuclear reaction, the nucleus bombarded does not remain largely intact while emitting small fragments of mass number 1 or 2, but splits into two or more reasonably equal portions This is *nuclear fission*, and occurs when U^{235} is bombarded with slow neutrons In splitting into such fragments as nuclei of barium, technetium, and so on, more neutrons are produced and if these are slowed by the inclusion of such "moderators" as heavy water in the system, more uranium atoms are split and a cyclic process is set up in which in extraordinarily short periods extraordinarily huge quantities of energy are released The application of such chain reactions to the development of the atom bomb is known to all

Artificial Radioactivity

In some nuclear reactions well known naturally occurring isotopes are created or "synthesized" in the process In the first reaction listed Mg^{26} is formed, a respectable magnesium isotope occurring as 11 1 per cent of all magnesium atoms In the second reaction, however, C^{14} is formed, and Cl^{38} is formed in the last—neither of these being known to occur in nature in more than traces Many such "unnatural" isotopes have been artificially formed and in all cases they were found to be unstable, and to rearrange spontaneously into more stable nuclei

Thus, C^{14} emits an electron to ... o ... nitrogen isotope N^{14} *The half life of this reaction is* ... The ... an electron to become the stable ... is ... li ... reaction is 38 5 minutes Such spo... ...to that of the spontaneous dec... ...nomenon is therefore known ... known

Radioactive ... for every chem... ...n ... been At least fifteen

described Artificial radioactivity generally involves the emission of beta particles and gamma rays Alpha particles are confined to those complex atoms with mass numbers of 190 or more, with the single exception of one naturally occurring samarium isotope, Sm^{157}, which emits the weakest known alpha rays and has a half life of the order of two hundred billion years

A great many radioactive isotopes have found use in one branch or another of biochemical research Kamen (1) lists 201 of them distributed among 46 different elements Those radioactive isotopes most used in metabolism studies are listed in table 47, the data again being taken from Sullivan (2) The choice between several possible radioactive isotopes depends upon half life, characteristic radiation, the ease of manufacture, and so on Details on the preparation of these isotopes and their comparative uses may be found in great detail in Kamen

It will be noted that no radioactive isotopes of more than very fugitive life have been found for two important elements, nitrogen and oxygen and it is not expected that any will be found in the future

METABOLIC STUDIES

In this book several applications of isotopes to biochemistry have been mentioned Thus in Chapter 2 we spoke of the isotope dilution technique as applied to analytical biochemistry, where an isotopically "tagged" substance is added to a mixture containing the normal substance Separation of a pure sample of the substance and the determination of the fraction which is isotopically tagged allows us to compute the original total quantity of the substance in the mixture In Chapter 8 we referred to the application of radioactive isotopes in cancer therapy

The most important application of isotope technique to the science of biochemistry, however, involves the elucidation of intermediary metabolic pathways We know with considerable accuracy the nature of the substances ingested by man and how they are broken down in the alimentary canal prior to absorption We know also the final materials which man biosynthesizes out of these absorbed foodstuff derivatives and the waste products excreted in the urine But how, exactly, do the foodstuffs pass from glucose and amino acids to steroids and urea—by exactly what steps—through exactly which intermediate stages?

The simplest attempt at a solution has been to feed an animal (when possible, a human) measured quantities of a particular chemical, and observe any increase in some component of the urine This would give a first stage and a last stage, with only conjecture as to the nature of the many possible intermediate steps Even if it were possible to take animals which had fed on substance A, kill them at various intervals after ingestion and

[illegible] organ for all its components, such [illegible] to be considered that it would be [illegible] assemble such a jigsaw puzzle

[illegible]

[illegible] used in metabolic studies

[illegible]	MASS NUMBER	HALF LIFE
[illegible]	[illegible]	11 years
[illegible]	[illegible]	20 minutes
[illegible]	[illegible]	5700 years
[illegible]	[illegible]	2 hours
[illegible]	[illegible]	2 6 years
[illegible]	[illegible]	15 hours
[illegible]	[illegible]	14 3 days
[illegible]	[illegible]	[illegible] days
[illegible]	[illegible]	33 minutes
[illegible]	[illegible]	2 million years
[illegible]	[illegible]	37 5 minutes
[illegible]	[illegible]	0 5 billion years
[illegible]	42	12 5 hours
[illegible]	43	22 5 hours
[illegible]	[illegible]	152 days
[illegible]	[illegible]	7 days
[illegible]	49	2 5 hours
[illegible]	52	[illegible] 8 hours
[illegible]	55	4 years
[illegible]	[illegible]	45 days
[illegible]	[illegible]	18 hours
[illegible]	56	[illegible] days
[illegible]	57	[illegible] days
[illegible]	[illegible]	[illegible] days
[illegible]	60	[illegible] years
[illegible]	61	3 4 hours
[illegible]	64	12 [illegible] hours
[illegible]	[illegible]	58 hours
[illegible]	[illegible]	[illegible] 5 hours
[illegible]	[illegible]	[illegible] days
[illegible]	[illegible]	[illegible] hours
[illegible]	[illegible]	[illegible] days
[illegible]	[illegible]	[illegible] days
[illegible]	121	[illegible] days
[illegible]	[illegible]	[illegible] days
[illegible]	126	[illegible] years
Iod ne	129	
Iod ne	130	
Iod ne		
I d ne		
Iod ne		

It then occurred to biochemists to 'label' an ingested compound, that is add to its structure some chemically identified group by means of which its wanderings through the cell mechanisms of man might be traced. Before the days of widespread application of isotopic technique, such a label might include a particular metal or compound not ordinarily found in the body, such as lead or benzoic acid. These materials would be all "label." Such experiments sometimes had interesting results. Thus ingestion of benzoic acid resulted in the excretion in the urine of hippuric acid, a condensation product of benzoic acid and the amino acid, glycine. Such studies pointed up the use of glycine as a "detoxifying" substance.

Another example of such labeling is the study of Knoop of the fat metabolism of animals through their ingestion of various phenyl derivatives of fatty acids (see page 519). It was possible to locate in the urine certain phenylated compounds such as hippuric acid or phenylacetylglycine and from their occurrence advance a theory as to the beta oxidation of fatty acids by the body.

The fault in all such studies is that the materials ingested are entirely or in part non physiological. Since it is the essence of this labeling process that the label be a group which can be recognized by the chemist from among the myriad groups already present in the body, as a necessity it must be something the body does not ordinarily encounter. The result is that biochemists find themselves studying abnormal metabolism and the detoxication of poisons, not the normal processes they most wish to study.

The problem was, then, to find a substance which to the body was normal and indistinguishable from its usual raw materials, and yet which to the chemist was surely identifiable under all circumstances. This proposition, impossible on the face of it, was solved neatly and handily by the use of isotopes.

Earliest experiments were conducted with naturally occurring stable isotopes. In the early 1930's ways were discovered for fractionating elements or simple compounds so as to obtain fractions richer than normal in one of the less common isotopes. Such concentrated sources of, say, deuterium or N^{15}, could be used in the synthesis of fatty acids or amino acids which, in turn, would be richer than normal in the rare isotope. If animals were fed these isotopically tagged substances and later killed and their organs analyzed, it would no longer be a question of trying to place all the compounds found, but only those which likewise were richer than normal in the ingested isotope. The jigsaw puzzle is tremendously simplified.

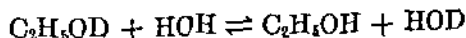
Except for those elements, notably nitrogen and oxygen, where usable radioactive isotopes do not exist, the use of stable isotopes has given way to that of the radioactive variety. This is so for one important reason—radioactive isotopes are far easier to estimate in far smaller quantities than

are the stable isotopes The stable isotopes can be estimated by means of the *mass spectrograph*, which operates on the principle that in an electric field and magnetic field of given intensities, two ions of similar charge will be deflected by an amount inversely proportional to their masses Thus hydrogen ions travel in a path more curved than that of deuterium ions and therefore strike the photographic plate at a different point Mass spectro analysis is expensive Radioactive isotopes, on the other hand, can be meas ured by means of their radiations, which can be made to actuate counting devices

The use of radioactive isotopes carries with it one important disadvan tage Because of the potential damage to living tissue by their radiations, unusual precautions must be taken which increase both the expense and the inconvenience of the procedure In conducting experiments with tracers, whether radioactive or stable, several precautions must be taken

(1) Sufficient isotope must be used to remain detectable after dilution in the body Once introduced into the body, glycine containing C^{14}, for instance, becomes indistinguishable from all other glycine in the body and in not too long a time becomes evenly distributed throughout all the body glycine Subsequent isolation of glycine from some organ of the animal will then contain only a fraction of the C^{14} contained by an equivalent mass of the starting material This smaller amount must still be detectable or the initial concentration must be increased

(2) The tracer used must be located on the molecule in a stable fashion and must not "exchange" with its environment Thus, if ethyl alcohol is prepared with a deuterium atom bound to the oxygen in the place of the normal hydrogen thus C_2H_5OD, in the hope that ingestion of the compound will show the metabolic fate of the alcohol group, non significant results would be obtained The reason for this is that if C_2H_5OD were simply allowed to remain in water solution it would be found that there was a rapid exchange of oxygen bound hydrogen (or deuterium) between the alco hol and the water In the body, therefore, the deuterium of the ethyl alcohol



$$C_2H_5OD + HOH \rightleftharpoons C_2H_5OH + HOD$$

would quickly be distributed throughout the body water and any organic compound in which it might later be identified might just as *well have* acquired it from water as from alcohol In general, deuterium bound to oxygen, nitrogen, or to a carbon adjacent to a carbonyl group, suffers from this instability and can not be used in tracer experiments Deuterium bound to carbons not adjacent to carbonyl groups stays put and may be used

(3) Abnormalities in metabolism must not be brought about by the use of the isotopes Radiation may alter the functioning of tissues and radioac

the isotopes must therefore be used in the lowest concentrations consistent with their sure analysis With stable isotopes this does not occur but there is a sufficiently large percentage difference between deuterium and its normal isomer to allow perceptible changes in reaction rates which must be considered In high concentrations, deuterium therefore inhibits respiration and fermentation

(4) Allowance must be made for the loss in intensity of isotope radiation during the time of the experiment and the time required afterwards for analysis The loss of activity of C^{14} is not significant in ordinary experiments since it has a half life of 5700 years On the contrary C^{11} has a half life of 20 minutes and 99 9 per cent of its activity is lost in 3 5 hours

DYNAMIC STATE OF BODY CONSTITUENTS

The paths or partial paths of many biosyntheses have been determined by means of isotopic techniques Thus it was found that glycine is used as one of the building blocks for heme and that acetic acid is an important precursor of the steroid nucleus In Chapter 7 the precursors for each atom of uric acid were given as determined by isotope studies Various carbons were found to arise from carbonate, formate and lactate ions one of the nitrogens with two neighboring carbons arose from glycine while the remaining two nitrogens were stated to be derived from the nitrogen pool

It is the nature of this nitrogen pool or more generally the *metabolic pool*, that was the most significant contribution of isotopic techniques to the understanding of intermediary metabolism One of the earliest findings once isotopes began to be used was that the compounds of the body were anything but stable The depot fat for instance had been thought to lie quiescent within its cells never stirring except and until undue exertion or underfeeding required its mobilization This is not the case If an organism is fed on deuterium-containing fat, the deuterium is eventually found to occur uniformly through the depot fat This is not because of isotope exchange as described earlier in the case of oxygen bound deuterium Here the deuterium was bound stably to carbon and the only hypothesis that fits the fact is that the molecules of fat within the stores of the body are constantly changing with incoming fat in a continuous dynamic equilibrium

This is even more dramatically demonstrated with experiments carried out with amino acids labeled with heavy nitrogen (N^{15}) Here the nitrogen isotope, fed as part of one amino acid was quickly found to occur in greater or lesser degree among all the kinds of amino acids in the body (with the exception of threonine and lysine) The picture one perceives is of a ceaseless deamination and reamination of most amino acids

It would seem that this dynamic equilibrium is a great waste of energy,

that it would be more economical on the part of living matter to allow its *protein, fat, and other constituents to remain fixed* in position. Probably this idea of "rest" is a false one. Actually, the molecules with which the body deals are, of necessity, so complex that they lack sufficient inherent stability to "rest", just as the long rod balanced on the nose is too unstable to remain standing if the juggler stops moving his head in a calculated fashion. Their instability is such that they are forever falling apart and only a device whereby they are put together again at an equivalent rate can allow the body to make use of their tremendously complicated structure. Since the larger molecules which compose living tissue are constantly releasing smaller molecules and groups, such fragments are always available as raw materials for biosyntheses and are spoken of as the metabolic pool. It is in this sense that two of the nitrogens of uric acid are said to be obtained from the "nitrogen pool".

Isotopes have also been used to study the permeability of certain cell membranes to specific ions, the volume of the intracellular and extracellular spaces in the body, the blood circulation time, uptake and retention of specific materials by various tissues, the half life of erythrocytes, and so on. From all current indications the uses and usefulness of isotope techniques will increase steadily in the future and a continually greater fraction of future biochemical literature will be devoted to a description of their uses and an elucidation of their findings.

REFERENCES

1. KAMEN, M. D. *Radioactive Tracers in Biology*. 2nd edit. New York, Academic Press, 1951.
2. SULLIVAN, W. H. *Trilinear Chart of Nuclear Species*. New York, John Wiley and Sons, 1949.

APPENDIX III

Principles of Thermodynamics

Active animal life is possible only if we have some way of converting the chemical energy of foodstuffs into work. Thermodynamics is the subject which grew out of the systematic study of the problems of the interconversion of work and heat primarily based on the heat engines which are the most characteristic feature of our modern industrialized civilization. The laws of thermodynamics apply also to electrochemical cells and to chemical machines such as the human body.

Most books on thermodynamics are written for physical chemists and engineers, and consequently employ the shorthand language of mathematics freely. For engineering calculations mathematics is indeed indispensable, but we shall see that merely for an understanding of the basic principles, which is all we need here, not much mathematics is needed.

All that readers of this book will need, we believe, is a fairly clear notion of the two basic laws of thermodynamics and the meaning of two thermodynamic concepts which prove useful in thinking about the energetics of biological systems as well as about heat engines and other machines used in engineering.

There are sometimes said to be three laws of thermodynamics, but we shall need only the first two here.

1. The first law is so well known and universally accepted that it will hardly cause any difficulty. It is simply the familiar law of the conservation of energy, and states that in the process of converting heat into work, chemical energy into work, or in any energy conversion, energy can neither be created nor destroyed. If a handful of coal, or a cheese sandwich, is considered, we know that it contains just so much chemical energy, and that no chemical machine or heat engine, however ingenious, will enable us to get more than the mechanical equivalent of that energy out of it. (Of course we live in an age in which certain elements of matter can be transformed, to a very limited degree, into energy and such operations are excepted from the law as we have stated it. But for matter-energy trans-

formations there is a more general law whose statement includes both matter and energy, this more general law will not concern us here)

From the first law it follows that no perpetual motion machine of the first type —that is, one getting energy from nowhere, or producing more energy than is fed into it—can ever be constructed

The total energy of a system is symbolized in English language text books as E, and is usually expressed in heat units such as calories

2 The second law is harder to state in a way which sounds so immediately obvious, although its validity is just as well established One way of stating it is that no work can be obtained from a heat engine which has no temperature difference between the highest temperature at which its working substance operates and the lowest temperature available to it For instance, the ocean has a mean temperature many degrees above the absolute zero, and we know that temperature is an expression of the kinetic energy of the molecules of the substance whose temperature we measure Consequently the ocean contains enormous stores of energy Nevertheless nothing is more certain than the fact that you can not devise a heat engine for an ocean liner or battleship which will drive the ship by utilizing the kinetic energy of the molecules of water in the ocean The trouble is that you do not have a sink" at a still lower temperature into which to dump your ocean water after it has gone through your engine

The reason this sink" is necessary is not clear to all students But consider the following analogy If you have in your possession a cylinder of compressed gas, say at about 80 pounds per square inch you can discharge this gas through a little turbine and make it do some work In space you could use the stored energy directly as a reaction motor which operates on the same principle as a rocket But suppose some Superman' puts you and your little cylinder down on a planet where the atmospheric pressure is 80 pounds per square inch—the same as in your cylinder If you then open the valve of your cylinder, nothing happens The compressed gas is still there with all its stored energy, but you no longer have a 'sink" of lower pressure No pressure gradient, no work Similarly with heat engines, no temperature gradient, no work

In the tropics where the surface temperature of the ocean may be much higher than that of the depths below, a temperature gradient is available and utilization of this has enabled work to be produced, although no machine capable of doing this has yet proved worth the financial investment (several million dollars) involved

The second law of thermodynamics involves when one attempts to formulate it at all precisely, the concept of entropy An increase in entropy means a loss of availability of the energy of the system under considera

tion This concept, unlike those of temperature, pressure heat content, and so on did not grow naturally from earlier concepts already more or less familiar to the ordinary man but was introduced to enable us to formulate precisely how much of the energy of a given system is available as work We have already seen (Chapter 17) how Sadi Carnot showed how to calculate the maximum amount of the heat introduced into a steam engine which could be converted into work This heat is called the *available heat*, and amounts to the fraction

$$(T_2 - T_1)\ T_2$$

of the total heat The remainder of the heat is the *unavailable heat* which although still a form of energy has to be discharged at the end of each cycle of the engine into the sink' (which may be the atmosphere or a water condensor) If a heat sink at absolute zero were available T_1 would be zero, and the engine could convert, ideally all of the heat of the steam into work Of course such a sink is not available at least on earth It has been shown that the proportion of the heat which is unavailable is measured by the change in entropy during the cycle

Many early physicists considered that in general entropy is increasing everywhere in the universe and that ultimately all forms of energy will be degraded into heat in a universe at thermal equilibrium From such heat the second law says no work can be obtained This has led some scientists, writing for the general public to call entropy time's arrow' It would involve an excursion too far into philosophy to consider whether or not this idea is valid for the universe as a whole and fortunately this problem does not concern us here All the transformations of energy studied by us on earth are, to some degree at least irreversible, and in an irreversible process entropy always increases So for the processes we have to deal with, entropy is truly time's arrow In some cases, we can connect our system to an outside source of energy and restore it to its original state But it will always be found that the net entropy of both systems has been increased So long as we restrict our attention to *closed* systems (and the idea of a closed system is not hard to grasp, although in actual practice complete insulation of any system from all others is extremely difficult), the second law of thermodynamics is one which can not be beaten

In order to define this important quantity entropy, we must consider the heat exchange and temperature of the system (we deliberately omit other considerations which would make the definitions more complicated) If a system absorbs a very small quantity of heat we may represent this microscopic quantity by the symbol q (there are reasons for not using the

symbol dq which might seem a better choice to students of the calculus) Represent the absolute temperature by T and the change in entropy by dS Then dS is defined by the relations

$$q/T_{max} < dS \text{ for any spontaneous process}$$

$$q/T_{min} > dS \text{ for any non spontaneous process}$$

$$q/T_{constant} = dS \text{ for any completely reversible process}$$

These equations and inequalities are another way of stating the second law of thermodynamics It is not a law which can be seen intuitively, but rather a generalization from the results of man's experience with machines What it really states is that it is impossible to construct a perpetual motion machine of the second type" A perpetual motion machine of the second type is one which receives and discharges its heat at the same temperature We have already tried to show how one can not expect such a machine to work Every layman now knows that perpetual motion machines have been declared impossible, but from time to time some would be inventor designs what is really a perpetual motion machine of the second type—without of course, calling it that

It will be obvious to those who have studied calculus that if we knew dS to be a complete differential, the third expression above could be integrated, and we could obtain an equation involving not the infinitesimal dS but S itself Even in such a case, it must be remembered that a constant of integration must be introduced so that S remains unknown to the extent of an arbitrary constant This does not matter for most work however, as we are usually interested merely in changes of entropy, where the arbitrary constant cancels out By making certain assumptions as to states of matter at which matter has zero entropy, the constant could be evaluated and absolute entropies calculated We shall not need such values here however, and the assumptions necessary introduce some new difficulties of their own

Our chief reason for introducing a mathematical symbol for entropy here is to be able to define the *free energy* a concept of the greatest value in thinking about the energetics of chemical change We write

$$G = H - TS$$

In this expression G stands for the free energy as defined by Gibbs, H stands for the heat content, and T and S are the absolute temperature and the entropy Some writers use the symbol F for the Gibbs free energy, but the symbol was originally used for a slightly different concept introduced by Helmholtz, and some confusion has resulted from the transfer of the symbol to the concept of free energy as we use it today Also we

shall want the symbol F for the *Faraday* (the amount of electricity transferred by one equivalent of a reactant in an electrochemical cell).

The units in which entropy is expressed are not independent of the units of temperature used, for we see from the above expression that the product TS must come out in energy units in order to be directly comparable with H. Similarly, G must be expressed in energy units. The usual units for S are calories per degree; the usual units for G are calories. In biochemistry we are more likely to use Kcal. instead of the calorie (one Kcal. equals 1000 calories).

The great importance of G stems from the fact that it is a measure of the driving force of a chemical reaction. If a reaction as we write it involves a decrease of free energy, that is, the combined free energies of the products is less than that of the reactants, thermodynamics predicts that this reaction will go spontaneously, although thermodynamics cannot predict the rate, and in the absence of the proper catalysts the reaction may go so slowly that we are quite unable to measure its rate. If the reaction as written involves an increase in G, then we know it will never go unless external energy is applied and it thus becomes part of a more complex total reaction in which there is a net decrease in free energy. This is a law of great generality and power, and no exceptions to it have ever been observed. A few brilliant writers have argued that certain at least of the processes of life violate the principle, but they have never been able to produce thermodynamic evidence.

We should mention that the free energy change of a reaction, Δ G, is also a function of the concentrations of the reagents and reactants, so that in the case of reactions involving a small Δ G, changing the concentration of one or more of the reactants may change the sign of Δ G and cause the reaction to run in the reverse direction. To allow for the concentration we have to make use of the "chemical potential" of the various chemical species with which we are dealing. The concept "chemical potential" was introduced by Gibbs and may be defined as the effect on the free energy of adding a small quantity of one of the constituents at constant temperature and pressure, the concentration of other constituents remaining constant. Mathematically, this is expressed by the relation—

$$\mu_i = (\partial G/\partial n_i)_{T,P,n_j}$$

where the round d's indicate partial differentiation, as usual in the calculus, G is the free energy, n_i the concentration of constituent i, and n_j the concentration of constituent j and so forth. The subscripts T, P, n_j and so forth, indicate the variables which are to be kept constant during the differentiation. Gibbs introduced the symbol μ for the chemical potential.

It will be seen without any mathematical knowledge that a quantity

symbol dq which might seem a better choice to students of the calculus) Represent the absolute temperature by T and the change in entropy by dS Then dS is defined by the relations

$$q/T_{max} < dS \text{ for any spontaneous process}$$

$$q/T_{min} > dS \text{ for any non spontaneous process}$$

$$q/T_{constant} = dS \text{ for any completely reversible process}$$

These equations and inequalities are another way of stating the second law of thermodynamics It is not a law which can be seen intuitively, but rather a generalization from the results of man's experience with machines What it really states is that it is impossible to construct a perpetual motion machine "of the second type" A perpetual motion machine of the second type is one which receives and discharges its heat at the same temperature We have already tried to show how one can not expect such a machine to work Every layman now knows that perpetual motion machines have been declared impossible, but from time to time some would be inventor designs what is really a perpetual motion machine of the second type—without, of course, calling it that

It will be obvious to those who have studied calculus that if we knew dS to be a complete differential, the third expression above could be integrated, and we could obtain an equation involving not the infinitesimal dS but S itself Even in such a case it must be remembered that a constant of integration must be introduced so that S remains unknown to the extent of an arbitrary constant This does not matter for most work however, as we are usually interested merely in changes of entropy, where the arbitrary constant cancels out By making certain assumptions as to states of matter at which matter has zero entropy, the constant could be evaluated, and absolute entropies calculated We shall not need such values here, however, and the assumptions necessary introduce some new difficulties of their own

Our chief reason for introducing a mathematical symbol for entropy here is to be able to define the *free energy* a concept of the greatest value in thinking about the energetics of chemical change We write

$$G = H - TS$$

In this expression G stands for the free energy as defined by Gibbs H stands for the heat content, and T and S are the absolute temperature and the entropy Some writers use the symbol F for the Gibbs free energy, but the symbol was originally used for a slightly different concept introduced by Helmholtz, and some confusion has resulted from the transfer of the symbol to the concept of free energy as we use it today Also, we

shall want the symbol F for the *Faraday* (the amount of electricity transferred by one equivalent of a reactant in an electrochemical cell)

The units in which entropy is expressed are not independent of the units of temperature used for we see from the above expression that the product TS must come out in energy units, in order to be directly comparable with H Similarly, G must be expressed in energy units The usual units for S are calories per degree, the usual units for G are calories In biochemistry we are more likely to use kcal instead of the calorie (one kcal equals 1000 calories)

The great importance of G stems from the fact that it is a measure of the driving force of a chemical reaction If a reaction as we write it involves a decrease of free energy that is, the combined free energies of the products is less than that of the reactants, thermodynamics predicts that this reaction will go spontaneously, although thermodynamics can not predict the rate, and in the absence of the proper catalysts the reaction may go so slowly that we are quite unable to measure its rate If the reaction as written involves an increase in G then we know it will never go, unless external energy is applied and it thus becomes part of a more complex total reaction in which there is a net decrease in free energy This is a law *of great generality and power and no exceptions to it have ever been* observed A few brilliant writers have argued that certain at least of the processes of life violate the principle, but they have never been able to produce thermodynamic evidence

We should mention that the free energy change of a reaction Δ G, is also a function of the concentrations of the reagents and reactants so that in the case of reactions involving a small Δ G changing the concentration of one or more of the reactants may change the sign of Δ G and cause the reaction to run in the reverse direction To allow for the concentration we have to make use of the "chemical potential" of the various chemical species with which we are dealing The concept "chemical potential" was *introduced* by Gibbs, and may *be defined as the effect on the free energy of* adding a small quantity of one of the constituents at constant temperature and pressure, the concentration of other constituents remaining constant Mathematically, this is expressed by the relation—

$$\mu_i = [\partial G/\partial n_i]_{T\,P\,n_j}$$

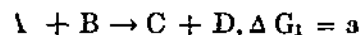
where the round d's indicate partial differentiation, as usual in the calculus, G is the free energy n_i the concentration of constituent i, and n_j the concentration of constituent j, and so forth The subscripts T, P, n_j and so forth, indicate the variables which are to be kept constant during the differentiation Gibbs introduced the symbol μ for the chemical potential

It will be seen without any mathematical knowledge that a quantity

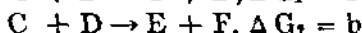
such as μ which measures the effect on the free energy of variations in concentration of a constituent of a system gives a measure of the driving force due to that constituent This value is the one employed in chemical calculations, and its use enables far reaching conclusions to be drawn and important relations such as the Gibbs phase rule to be deduced Such calculations are out of the scope of this book Examples will be found in any of the references

The free energy change, ΔG, of a chemical reaction represents the maximum energy which we can possibly get out of it, either in the form of work or of heat This will be discussed slightly more in detail later

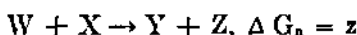
An important feature of free energy values is that they are additive If we know the free energies of the starting materials of a reaction, and the free energies of the final products, it does not matter through how many intermediate stages, or by what steps, the reaction proceeds, for the net free energy change will still be the same Consider the following hypothetical reactions



$$A + B \rightarrow C + D, \Delta G_1 = a$$



$$C + D \rightarrow E + F, \Delta G_2 = b$$

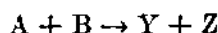


$$W + X \rightarrow Y + Z, \Delta G_n = z$$

then the sum

$$(a + b + \quad + z) = \Delta G$$

which is the free energy change of the reaction



$$A + B \rightarrow Y + Z$$

Early physical chemists thought that the heat of reaction measured the tendency of the reaction to go, but studies on electrochemical cells showed this was not quite true One chemical cell at least (the lead calomel cell) actually absorbs heat during its operation and the net work obtainable is greater than the heat of the reaction But in all cases it is found that ΔG is the proper indicator of whether a reaction will go, and a measure of the maximum amount of energy which can be obtained, whether as heat or in some other form

For an electrochemical cell ΔG may be calculated from the voltage and the number of equivalents of electricity transferred per mol The equation is

$$\Delta G = -n_e FV$$

where G represents the free energy, n_e the number of equivalents of electricity per mol, F the value of the Faraday, and V the voltage of the cell

We are so accustomed to heat engines in daily life that we are likely to regard other methods of converting energy into work such as the battery powered motor or the animal body as exceptional and to feel surprised if they compete in mechanical efficiency with heat engines Actually there is no reason to think that burning a fuel and then converting as much of the resulting heat as possible into work in a heat engine makes the best use of the contained energy We have already seen in Chapter 17 that it sometimes does not To pursue this argument a little further let us take some actual figures (1) The heat of oxidation of metallic zinc in contact with a concentrated solution of zinc sulphate is about 55.2 kcal per mol of zinc consumed The electrical energy we can get from a cell employing this as one electrode is somewhat less (50.5 kcal per mol) Nevertheless we see that if we assume the heat utilized in one of our best heat engines with an efficiency of 40 per cent and the electrical energy utilized by an electric motor with an efficiency of 80 per cent we can get by utilizing the heat of reaction at the most 22.0 kcal per mol of zinc whereas our battery motor combination will give us 40.4 kcal or twice as much

REFERENCES

1 EUCKEN A et al *Fundamentals of Physical Chemistry* New York N Y McGraw Hill Book Co 1925

2 GIBBS J W *Collected Works* New York N Y Longmans Green & Co 1928

3 GUGGENHEIM E A *Modern Thermodynamics* London England Methuen & Co 1933

4 LEWIS G N AND RANDALL M *Thermodynamics* New York N Y McGraw Hill Book Co 1923

search into the mechanisms of organic reactions frequently postulate their existence as short lived intermediates

Positively charged hydrogen containing ions are not amphoteric They act only as acids, since the tendency to accept a second hydrogen ion is negligible Molecules or ions that do not contain hydrogen can not, obviously, act as acids as they have no hydrogen ion to lose They may either act as bases only, as in the case of chloride ion or carbonate ion, or they may be neither acids nor bases, as for instance carbon tetrachloride

(3) Sodium hydroxide is not a base Sodium hydroxide is actually a stoichiometric mixture of sodium ions and hydroxyl ions The hydroxyl ion is the base, because of its strong tendency to accept a hydrogen ion and become water Sodium ion is neither an acid nor a base

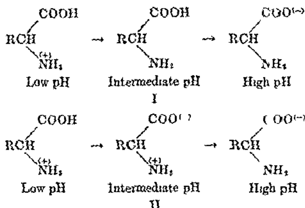
The great generalization of the Brønsted Lowry viewpoint of acids and bases is the removal of the hydroxyl ion from its position as *the* pre eminent base Ammonia is a base by virtue of the fact that it will accept a hydrogen ion and become an ammonium ion, *not* because it will form ammonium hydroxide in solution The only reason that the hydroxyl ion was ever accepted by chemists as being of special significance with respect to basicity was the accident that most chemical reactions have been studied in aqueous solutions Hydroxyl ion is the strongest base that can exist in aqueous solutions If a stronger base, one with a greater tendency to accept hydrogen ions such as amide ion, NH_2^-, were placed in water in the form of potassium amide, the NH_2^- would strip water of hydrogen ions, leaving hydroxyl ion behind and itself becoming ammonia An analogous situation would exist if any other "super base" were dissolved in water An apparent "hydrolysis" would take place (actually, a successful competition on the part of the "super base" for hydrogen) and only hydroxyl ion would be left—apparently *the* base If all reactions were studied in liquid ammonia, the amide ion would be *the* base, and if they were all studied in glacial acetic acid, acetate ion would play the role If a little sodium hydroxide is added to glacial acetic acid for instance, the hydroxyl ion will strip acetic acid of hydrogen to form water and leave acetate ion behind

A well buffered solution from this standpoint is one in which the competition for hydrogen ion on the part of hydroxyl ions and some other ion (acetate, acid phosphate, barbiturate) is a fairly even one If a small amount of hydroxyl ion is added to an equimolar mixture of acetic acid and acetate ion, then the weaker "protophilic" (hydrogen accepting) properties of acetate ion are sufficiently counterbalanced by its preponderant concentration (mass action law) to make the competition quite even The pH change with addition or subtraction of hydroxyl ion is therefore small In a poorly buffered solution the comparative protophilic properties of hydroxyl ions and the other ion present are so widely different that no practical difference

in concentration will make up for it Thus, if sodium hydroxide is added to an equimolar mixture of hydrochloric acid and sodium chloride, the basic properties of chloride ion are so weak that even in a vast concentration excess it can not prevent the added hydroxide from seizing every hydrogen ion it can hold Change of pH with added base or acid is therefore large

The Brønsted Lowry concept of acids and bases proves its usefulness in biochemistry in two ways in particular, (a) in the consideration of amino acid dipolar ions, and (b) in that of the buffer systems of the blood

The old fashioned concept of amino acid structures at varying pH was as shown in formula I Actually, this is *not* so The error arose chiefly from the perhaps natural assumption that a cationic acid such as the RNH_3^+ ion must be stronger (i e have a greater tendency to lose a hydrogen ion)



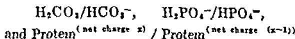
than a neutral acid such as RCOOH After all the cationic acid has an "extra" hydrogen ion The assumption is wrong, however It is the neutral acid in this case that is the stronger There is a definite intermediate pH for all the natural amino acids where the —COOH has lost its hydrogen ion while the $—NH_3^+$ still retains the one it possesses There is always a pH sufficiently basic so that both acid groups have lost their hydrogen ion The actual sequence of events is therefore as shown in formula II

In any consideration of the buffering power of blood, the situation is confused by the fact that the fossil remains of old fashioned theories are so permanently preserved in the clinical literature that it would actually do harm to attempt their removal What is said here is therefore intended only as a more modern view of the subject without any hope or even desire, that it will introduce a change in nomenclature

Such ions as Na^+ or K^+ are viewed by the clinician as "basic" because of a dim association with the so-called bases sodium hydroxide and potassium

hydroxide On the other hand, Cl^- is viewed as "acidic," again because it is reminiscent of hydrochloric acid Actually sodium and *potassium ions* are neither acids nor bases, and chloride ion, far from being an acid, is actually an exceedingly weak base (although at the pH of blood it is best regarded as neither acid nor base)

At the pH of blood, the only substances playing a role in acid base balance are various proteins (including hemoglobin, oxyhemoglobin, and



$$H_2CO_3/HCO_3^-, \quad H_2PO_4^-/HPO_4^{=},$$
$$\text{and Protein}^{(\text{net charge } x)} / \text{Protein}^{(\text{net charge } (x-1))}$$

III

plasma proteins), bicarbonate ion, carbonic acid primary phosphate ion, and secondary phosphate ion The conjugate acid base pairs involved are shown in formula *III* The role played by ions such as sodium, potassium, and chloride ion *as far as the buffering capacity of blood is concerned* is simply that of electrical make weights necessary to keep the blood as a whole at electroneutrality

For a discussion of still more general concepts of acid base relationships, the student is referred to Luder and Zuffanti (2)

REFERENCES

1 Brønsted J N The theory of acid and basic catalysis Trans Faraday Soc **24** 630–640, 1928
2 Luder W F, and Zuffanti S *The Electronic Theory of Acids and Bases* New York John Wiley & Sons Inc 1946

ABBREVIATIONS AND SYMBOLS

ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
A/G	Ratio of albumin to globulin
AMP	Adenosine monophosphate (Adenylic acid)
ATP	Adenosine triphosphate
BMR	Basal metabolic rate
CPP	Cyclopentanoperhydrophenanthrene
DFP	Di isopropylfluorophosphate
DNA	Desoxypentosenucleic acid
DOCA	Desoxycorticosterone acetate
DPN	Diphosphopyridine nucleotide
DRNA	Desoxyribosenucleic acid
FAD	Flavin adenine dinucleotide
FM	Flavin mononucleotide
FSH	Follicle stimulating hormone
HCG	Human chorionic gonadotrophin
HDP	Hexose diphosphate
ICSH	Interstitial cell stimulating hormone
Kcal	Large (or kilogram) Calorie
LH	Luteinizing hormone
NPN	Nonprotein nitrogen
PAH	p-Aminohippuric acid
PNA	Pentosenucleic acid
PSP	Phenolsulfonaphthalein
PTH	Parathyroid hormone
RNA	Ribosenucleic acid
RQ	Respiratory quotient
S_{20}	Sedimentation constant in svedbergs at 20°C
SDA	Specific dynamic action
TPN	Triphosphopyridine nucleotide
TSH	Thyroid stimulating hormone
VDM	Vasodepressor material

Index

E

F

G

I

J

K

U

V